Flow Cytometry Methods for DNA and Protein Analysis Using T-47D and T98G Human Cancer Cell Lines in Vitro

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M. Sc. Diploma Thesis

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SAMMENDRAG

I denne studien, var det to hovedmål: 1 - Å utvikle og etablere Vindeløv metoden for flowcytometrisk DNA-analyse, og deretter teste metoden på cellekulturer fra 2 cellelinjer utsatt for forskjellige behandlinger. 2 – Å utvikle og etablere en to parameter DNA- protein metode for flowcytometrisk målinger for å analysere både DNA innhold og protein mengden samtidig. Denne metoden ble også testet på cellekulturer med forskjellige forbehandlinger.

Den første metoden, Vindeløv, er en detergent - trypsin metode for å lysere celler og preparere kjernene for flowcytometrisk DNA-analyse. Denne metoden ignorerer mitokondrie-DNA og vurderer bare kjerne-DNA. Metoden bruker tre løsninger til farging; løsning A, B og C samt citratbuffer løsning. I tillegg til disse løsningene, brukes i den opprinnelige metoden PBS (1X) rense cellene. Denne metoden ble endret ved å endre eksponerings-tid, såpe type og konsentrasjon i løsning A samt utskifte PBS (1X) med 0,9% NaCl løsning. En kombinasjon av to såper, Tergitol og Triton - X 114 ble brukt til å erstatte NP-40 såpe brukt av Vindeløv i løsning A. Tiden i løsning A før løsning B ble tilsatt ble økt fra 10 minutter til 22 minutter.

Den andre metoden vurderer ikke bare kjerne-DNA, men også mitokondrie-DNA. Metoden tar tre dager. Første dag er fiksering av cellene i 50% etanol. Andre dag er farging av cellen i 0.1 mikrogram/ml FITC (fluorescein isothiocyanate) i mørke ved romtemperatur. Tredje dag er det farging først i 60 mikrogram/ml RNAase, og senere i 34 mikrogram / ml PI (propidium jodid) før flowcytometriske målinger starter.

Cellene som ble undersøkt var T-47D humane brystkreft celler og T98G humane hjerne kreft celler. Hver celle linje hadde sine derivater med forskjellig forbehandling. Disse cellene var:

• T98G LDRres (T98G lav doserate resistent): Celler som ble dyrket i [^{3}H]-medium med en spesifikk aktivitet av 1,67 μCi / ml i 10 måneder.

• T98G - P (T98G-Primed): Celler som ble bestrålt med 0,3 Gy / t i en time før de ble dyrket i 9 måneder uten stråling.

• T-47D LDRres (T-47D Low doserate resistente celler): Celler som hadde vokst i [^{3}H]-medium med en spesifikk aktivitet av 1,67 μCi / ml i 10 måneder

• T-47D - P (T-47-Primed): Celler som ble bestrålt med 0,3 Gy / t i en time før de ble dyrket i 4 år uten stråling.

• F10 (T-47D): Cellene som dyrket i [3H]-medium (0,46 ml [^{3}H] i 150 ml) med spesifikk aktivitet på 1,6 μCi / ml i 5 måneder og 13 dager og deretter dyrket i 17 dager uten stråling før de ble frosset ned 17.02.2003.

• F44 (T-47D): Celler som ble dyrket i 4% hypoksi og i [^{3}H]-medium med en spesifikk aktivitet på 1,67 μCi / ml i 35 dager og deretter dyrket i 45 dager uten hypoksi og stråling før de ble frosset ned 16.09.2009

Anvendelse av Vindeløv metoden til disse cellene ga følgende funn:

- T98G LDRes cellene hadde ca 30% mindre DNA-innhold i forhold til kontrollen, T-98G. Dette var det mest interesserante resultatetet av denne metoden.

- T-47D celler hadde ca 50 % mindre DNA –innhold i forhold til T98G celler.

- Behandling i 4% O_2 og i [^{3}H]-medium med en spesifikk aktivitet av 1,67 μCi/ml i 35 dager påvirket ikke DNA innholdet i F44 (T-47D) celler i forhold til cellene uten behandling i 4% O_2 .

- Kvaliteten på DNA histogrammer og de tilsvarende G1-CVs (ned til 3,27 ± 0,01) ga tillit til metoden.

Anvendelse av DNA-Protein metoden på cellene ga følgende funn:

- En sub-populasjon av T98G-P celler ble observert som hadde samme DNA innhold, men mindre mengde protein i forhold til normal populasjonen. Dette var det mest interessante resultatet av denne metoden.

- T98G LDRres celler hadde ca 30% mindre DNA-innhold i forhold til T98G kontroll celler.

- Fiksering av T98G celler enten med 50% eller 70% ethanol medførte ingen forskjell i cellesyklus distribusjoner.

- Øking FITC konsentrasjon fra 0.1μg/ml til 1 mikrogram/ml gav upålitelige resultater.

ABSTRACT

In the present study, there were two main goals: 1- To develop and establish the Vindeløv method for flow cytometric DNA analysis, and test the method by applying it to cells with different characteristics and backgrounds. 2- Top develop and establish a method which could analyse both DNA content and amount of protein at the same time which is a two parameter DNA – protein method for flow cytometric measurements. This method was also tested by applying it to cells with distinct characteristics and backgrounds.

The first method, Vindeløv, is a detergent – trypsin method for the preparation of nuclei for flow cytometric DNA analysis. This method ignores mitochondrial DNA and considers only nuclear DNA. The method uses three staining solutions; solution A, B, and C and citrate buffer solution. In addition to these solutions, the original method used PBS to clean the cells. This method was altered by changing mainly staining time, soap type and concentration in solution A as well as exchanging PBS with 0.9 % NaCl salt, a combination of two soaps, Tergitol and Triton – X 114 were used to replace NP-40 soap used by Vindeløv in solution A. The staining time in solution A before solution B was added was increased from 10 minutes to 22 minutes.

The second method considers not only nuclear DNA but also mitochondrial DNA. The method takes three days. First day is fixation of the cells in 50 % rectified spirit (ethanol) in cold. Second day is staining of the cell in0.1 µg/ml FITC (fluorescein isothiocyanate) in the dark at room temperature. Third day is the staining first in 60 µg/ml RNase, and later in 34 µg/ml PI (Propidium Iodide) before flow cytometric measurements start.

The cells which were investigated were T-47D human breast cancer cells and T98G human brain cancer cells. Each cell line had its derivatives with distinct backgrounds. These cells were:

- *T98G LDRres (T98G Low Dose Rate resistant):* The cells which grown in [^{3}H]-medium with a specific activity of 1.67 µCi/ml for 10 months
- *T98G – P (T98G -Primed):* The cells which were irradiated with 0.3 Gy/h during 1 hour and then grown for 9 months without radiation.
- *T-47D LDRres (T-47D Low Dose Rate resistant cells):* The cells which were growth in [^{3}H]-medium with a specific activity of 1.67 µCi/ml for 10 months
- *T-47D – P (T-47D-Primed):* The cells which were irradiated with 0.3 Gy/h during 1 hour and then grown for 4 years without radiation.
- *F10 (T-47D):* The cells which were grown in [^{3}H]-medium (0.46 ml [^{3}H] in 150 ml) with a specific activity of 1.6 µCi/ml for 5 months and 13 days. Hereafter they were grown 17 days without radiation before they were frozen on 17.02.2003.
- *F44 (T-47D):* The cells which were grown in 4% hypoxia and in [^{3}H]-medium with a specific activity of 1.67 µCi/ml for 35 days. Hereafter they were grown for 45 days without hypoxia and irradiation until they were frozen on 16.09.2009.

Applying the Vindeløv method to these cells gave the following findings:

- T98G LDRes cells had approximately 30 % less DNA content compared to its control, T-98G. This was the most interesting result of this method.

- T-47D cells had nearly half the DNA content compared to that of T98G cells.

- The treatment of growing in 4% O_2 and in [^{3}H]-medium with a specific activity of 1.67 µCi/ml for 35 days did not affect the DNA content of the cells compared to that of the cells without 4% O_2 treatment.

- The quality of the DNA histograms and the corresponding G1_CVs (down to 3.27 ± 0.01) increased confidence to rely on the method.

Applying the two-parametric DNA -Protein method to these cells gave the following findings:

- A sub population of T98G-P cells was observed which had the same DNA content but less amount of protein compared to wild-type T98G-P cells. This was the most interesting result of this project.

- As also found by the Vindeløv method T98G LDRres cells had approximately 30 % less DNA content compared to T98G control cells.

- Fixation of T98G cells either with 50 % or 70 % rectified spirit resulted in no difference in the cell cycle distributions.

- Increasing FITC concentration from 0.1µg/ml to 1 µg/ml gave unreliable results.

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1 INTRODUCTION

Cancer is a general name used for more than 100 diseases caused by abnormal cells growing out of control[1]. A cancer cell is defined by two heritable properties: 1- it reproduces in defiance of the normal restraints on cell growth and division, 2- it invades and colonizes other cells' territories[2]. A clump of such cells is called a *malignant tumour*. Cancer will kill 20% of mankind[2], which means approximately 1 person in each family will die because of cancer.
When one is exposed to radiation, cancer incident falls dramatically with age, that is, children are very radiosensitive whereas adults are quite resistant, for example, in case of thyroid cancer. It is also reported that females are more radiosensitive than males [1].

Treatment of cancer using radiotherapy can be performed both externally (long distance from tumour, known as *teletherapy*) and internally (short distance from tumour, known as *brachytherapy*). During teletherapy, a radiation source is directed onto the cell tissue to inactivate the tumour but normal tissue cells which are in the penumbra, receive a radiation dose as well. In case of brachytherapy, a radiation source is either loaded within body cavities (intracavitary) or implanted into a tumour tissue (interstitial)[3]. By using brachytherapy, it is possible to create a high dose in tumours whilst producing a low dose in normal tissue.

Radiation affects cell cycle distribution by inducing checkpoints which may lead to delays due to reparation of damages.

The more one knows about cells, the faster and easier one can find the cure(s) and defeat cancer. Cells have a molecule called *Deoxyribonucleic acid* (DNA) inside their nuclei. All information about the cell is inside DNA. What I did during this project was:

1. To establish and test the preparation method for flow cytometric DNA measurement, which was developed *by Lars L. Vindeløv* in 1982 in Copenhagen, to obtain nuclei from whole cells.

2. To establish and test a method to investigate protein content versus the content of DNA.

The first method, *Vindeløv*, shortly is about how to get rid of everything around the cell nucleus especially mitochondrial DNA. The goal is to have just nuclei so that a nucleic acid dye, Propidiumiodide (PI), can easily bind itself to DNA. After this step one can use a proper laser beam to get signals from the dye which will be proportional to the amount of the DNA. When one manages to obtain nuclei, then it is possible either to use the nuclei for further experiments to gain more information about DNA or let the dye bind itself to DNA in order to measure the content of the DNA.

The second method is about how to measure the amount of protein versus the amount of DNA.

Biophysics Group at University of Oslo investigates effects of Low Dose Irradiation. In this thesis, cell cycle distribution, the content of DNA, and protein content were investigated in:

- Cells exposed to external LDR ^{60}Co γ – irradiation for 1 hour – a treatment which has shown to remove HRS.
- Cells exposed to chronic β – irradiation from incorporated tritiated valine. These cells nearly died out but a subpopulation survived and repopulated.

2 THEORETICAL PART

2.1 Cell Biology

According to the *cell theory*[4]:

- Every organism is composed of one cell or more cells.
- The cell is the smallest unit having the properties of life.
- All cells come from pre-existing cells.

Cells are classified into two basic kinds, namely **prokaryotic** and **eukaryotic** cells. Prokaryotic cells are mostly bacteria and have no nucleus bound by a membrane. Their DNA is usually organized in a form of one chromosome and it moves freely in the *cytoplasm*.

On the other hand, eukaryotic cells, including human cells, have a nucleus bound by a membrane. The DNA, which is organized in the form of many chromosomes, is inside the nucleus.

2.1.1 The Cell Cycle

2.1.1.1 Introduction

Cells divide themselves in order to grow, to replace the dead cells with the new ones and to produce gender/germ cells. There are three different types of cell division, *binary fission* (in prokaryotic cells), *mitosis* and *meiosis*.

Due to the appearance of the cells in microscope, the cell cycle has been divided into two main phases, ***interphase***, where no difference in the cells is observed, and ***mitosis***, where dramatic changes start. Figure 2.1 shows a generalized cell cycle. The cell cycle starts every time a new cell is produced and it ends when the cell completes its own division. The time between two divisions is called the *mitotic cycle time* or more commonly known as *the cell cycle time* (T_C)[1].

2.1.1.2 Interphase

During *interphase* the cell grows, accumulating nutrients needed for the mitosis and duplicating it's DNA. The interphase lasts from almost 12 hours in the fastest stem cells, to the days and weeks maybe even to years in some type of cells. It is divided into three sub-phases/periods.

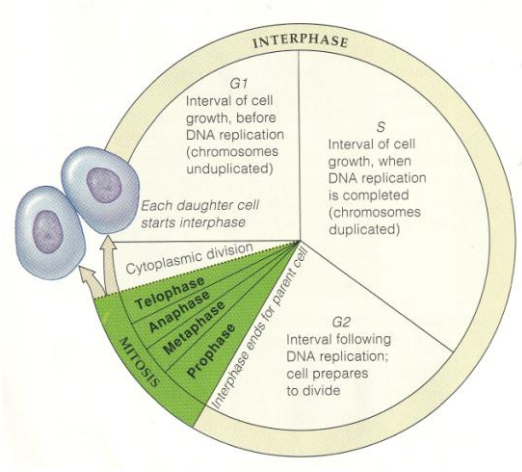


Figure 2.1: *Generalized cell cycle. The duration of each phase differs among different types[4].*

2.1.1.2.1 Gap 1 (G_1)

This is the gap, between the end of the mitosis and the start of the DNA synthesis. Before it was thought as a "pause". But now it is known that both G_1 and G_2 have important check-points for the cell cycle. Most cells need much more time to grow and double their mass of proteins and organelles than they need to duplicate their chromosomes and divide. That is why these gaps are necessary so that cells can double everything they need before the cell division. During G_1 the cell increases in size and the chromosomes are unduplicated. In the end of this gap there is a checkpoint which controls if the cell is ready to enter cell cycle and DNA synthesis. The length of G_1 can vary greatly depending on external conditions and extracellular signals from other cells.

2.1.1.2.2 DNA Synthesis (S)

This is the S (Synthesis) phase of the interphase. It requires 10-12 hours and takes about half of the cell – cycle time in a typical mammalian cell[2]. DNA molecules are the only molecules in the cells which can make a copy of themselves. DNA replication is quite similar to the process of producing pre- mRNA which is used in protein synthesis.

The replication starts with the enzyme *DNA polymerase* binding to specific places on the DNA molecule. Thereafter the hydrogen bonds between nitrogen bases in the two chains are broken. Free nucleotides are bound to nitrogen bases by the DNA polymerase properly. This process lasts a couple of hours even though almost 6 billion base-pairs are needed to be copied[6].

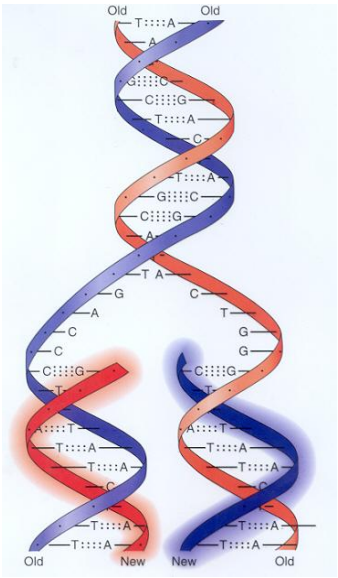


Figure 2.2: *Illustration of DNA replication[5].*

Many DNA polymerase enzymes work together at the same time so replication time gets shorter. There is almost 1 mistake per billion base-pairs during this process. Even if this is an extremely small number, continuously enzymes, find and repair the mistakes. The cell gets serious problems when there is mutation in the genes which code for these enzymes. This can causes the cell to re-produce itself and lead to the cancer.

2.1.1.2.3 Gap 2 (G_2)

G_2 represents the gap between the end of the DNA synthesis and the beginning of the next mitosis. The cell continues growing and it prepares itself to enter the mitosis phase which starts after G_2. Here in the end of the G_2, just before the mitosis, there is another checkpoint, *G_2/M checkpoint*, which controls if the cell is ready to enter mitosis.

2.1.1.3 Mitosis

The mitosis (also known, M phase) is the phase when the cell divides into two distinct cells, often called "daughter cells". Mitosis starts after the DNA replication. It lasts less than an hour in a mammalian cell[2] and it has five main stages or phases. They are *prophase*, *pro-metaphase*, *metaphase*, *anaphas*e, and *telophase*.

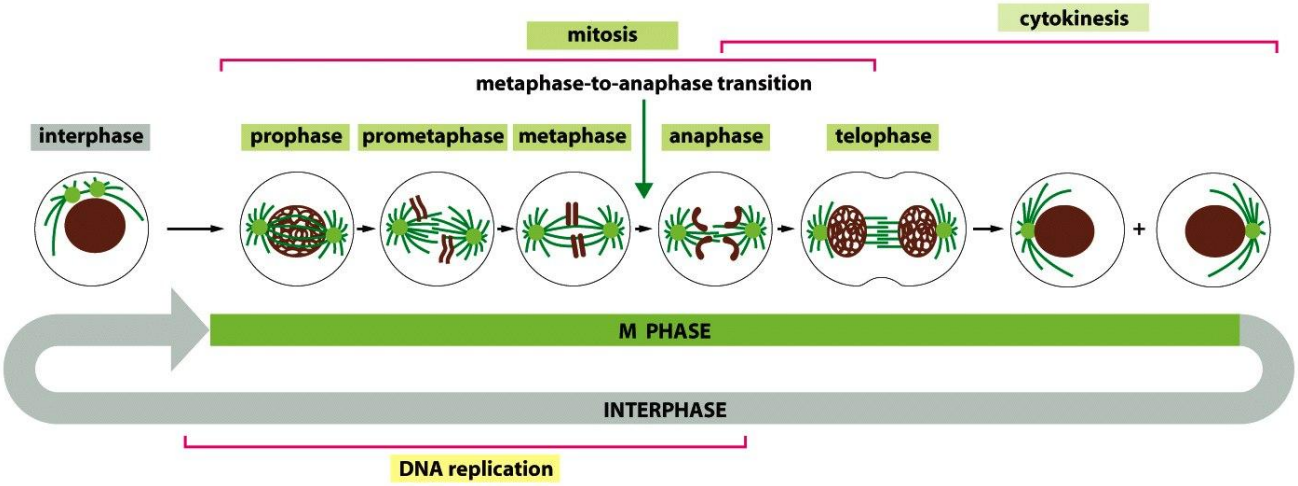


Figure 2.3: *The cell cycle with schematic phases of mitosis[2].*

In a mature human body there are almost 100 trillion cells[6] and all the time some of these cells die (many millions of cells every second[2]) and replaced by new cells. But some cells such as nerve cells, heart- muscle cells, egg-cells, cannot be replaced after birth. During DNA replication, 23 pairs of *homologous* chromosomes are duplicated. Homologous chromosomes essentially code for the same genes. Chromosomes are in the form of *chromatin* which is a complex combination of DNA and proteins that make up chromosomes. Two identical chromatids making up a replicated chromosome, are joined by *centromeres* for the process of cell division.

If the cells stop progressing through the cycle, they are said to be in G_0[1].

2.1.2 Regulation of the Cell Cycle

When cells go through the cell cycle, many things must be controlled such as if the cell is ready for the DNA synthesis, if extracellular and intracellular conditions are normal like temperature, or if there is any DNA Double –Strand Breaks (DSBs).

To regulate the cell cycle and cell number, cells have a main control system. The cell-cycle control system is a system which is based on a connected series of biochemical switches, each of which initiates a specific cell-cycle event. The switches are usually binary (on/off) and launch events in a complete, irreversible fashion. The cell-cycle control system is quite robust, reliable and highly adaptable and in most cells it can be modified to suit specific cell types and to respond to specific intracellular or extracellular signals[2]. Extracellular signal molecules which regulate cell cycle are divided into three groups[2]:

- *Mitogens* which bind themselves to surface receptors of the cell to trigger production of proteins, like *Myc*, to cause the cell cycle to start/continue.
- *Growth factors,* which stimulate cell growth (an increase in mass) by promoting the synthesis of proteins and other macromolecules and by inhibiting their degradation.
- *Survival factors*, which promote cell survival by suppressing the form of programmed cell death known as *apoptosis*.

These are required for the cell cycle to start/continue. On the other hand, cells have the system which orders the cell cycle to stop/repair. Cells use tumour –suppressing genes which code for some proteins such as *p53*, *retinoblastoma protein (Rb),* to block the cell cycle. Figure 2.4 summarizes the control system a cell uses. According to [1], there are three principal places in the cell cycle at which checkpoints function:

1. G1/S checkpoint
2. S phase checkpoint
3. G2/M checkpoint

According to [2], there are four important checkpoints; G_O, G_1, G_2, and M. Before the G_1 checkpoint (start/restriction point), cells depending on external signals may take several paths: They may progress, differentiate, senesce, or die[1].

After the G_1 restriction point, cells will enter S phase and no longer respond to altered growth conditions. If the cells have DNA damage before G_1 restriction point, they will be arrested in G_1 and p53 tumour suppressor gene plays the key role in this arrest. If the DNA damage occurs after G_1 restriction point, the cells will transiently be arrested in the S phase. If cells have DNA damage when they come to G_2 checkpoint, they will be arrested. This will allow the cells to have enough time to repair their damages. After this checkpoint, the control system will trigger the early mitotic events that lead to chromosome condensation. The cells which successfully progress into mitosis will be controlled by M checkpoint (metaphase-to-anaphase transition). If all the chromosomes are attached to the spindle, the control system stimulates sister-chromatid separation, leading to the completion of mitosis and cytokinesis.

Extracellular signals can stimulate a cell to enter the cell cycle from quiescence, G_0. Most cells in human body are in G_0 and but the molecular basis and reversibility of G_0 state differ in different cell types. For example most neuron and skeletal muscle cells are in a terminally differentiated G_0 state[2] which means the control system is completely dismantled.

To regulate the cycle, the control system uses two special types of proteins. They are called *Cyclin – dependent –kinases (Cdks)* and *cyclins*. Nobel Prize in Physiology or Medicine in 2001 was given due to the discovery of these key classes of regulatory proteins. There are several Cdks and cyclins. Cdks levels remain constant during the cycle, but each cdk must bind to a specific cyclin in order to be activated. Each cyclin is produced when it is needed and it is degraded when it has fulfilled its task, which is why cyclin levels oscillate during the cell cycle.

A good example about how cell cycle is blocked is shown in Figure 2.5. A DNA DSB is noticed which leads to activation of p53 by phosphorylation. p53 binds itself to the gene which codes for p21 and p21 arrests the cell cycle by inactivating the active G_1/S-Cdk and S-Cdk. It is important to note that at least half of all human cancers occur due to mutations in p53 gene[2].

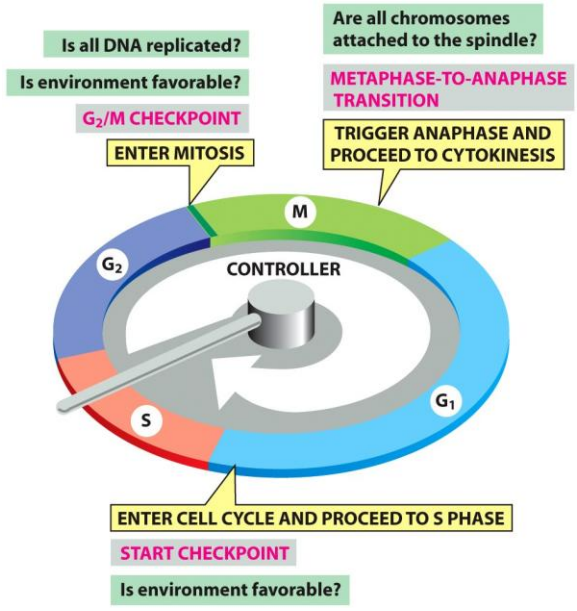


Figure 2.4: *The cell-cycle control system[2].*

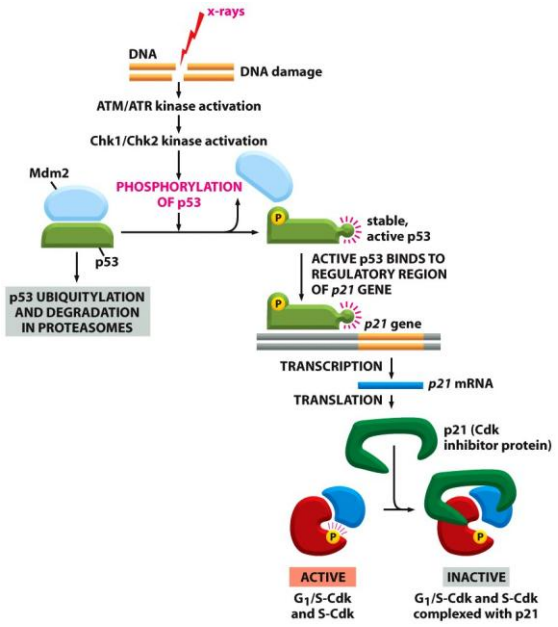


Figure 2.5: *How DNA DSB damage arrests the cell cycle in* G_1 *[2].*

Finally, it is important to mention that the cell lines used in the experiments are cultured cells in *vitro*. The cells are capable to divide themselves continuously because of genetic changes as long as the growth factors are favourable.

2.2 Radiation Physics

2.2.1 Radioactive Decay

When a radioactive nucleus disintegrates, the released energy can be in the form of particles (α-particles, β- particles, neutrons) and/or electromagnetic radiation (photons with different energies, γ- and x- rays).

The number of nuclei, *dN*, which will decay during the time interval from *t* to *t* + *dt*, is proportional to the number of radioactive nuclei, *N*:

$$-dN \propto N dt \tag{2.1}$$

Equation (2.1) can be written as:

$$\frac{dN}{N} = -\lambda dt \tag{2.2}$$

where λ is called the *decay constant*. By solving equation (2.2):

$$N = N_0 e^{-\lambda t} \tag{2.3}$$

equation (2.3), which is also known as the *Radioactive Decay Law*, can be obtained where N_0 is the initial number of radioactive nuclei [7].The rate of decay, known as *activity (A)*, can be written:

$$A = \frac{dN}{dt} = -\lambda N \tag{2.4}$$

Activity, *number of decays per unit time*, is measured in Becquerel (Bq) – *decays per second*- or in Curie (Ci) where *1 Ci is 3.7 x* 10^{10} *Bq*. By replacing $N = N_0/2$ in equation (2.3) and taking *ln* of both sides, one can obtain the half- life of a radioactive element ($t_{1/2}$), which is defined as the time elapsed before the intensity of the radiation is reduced to one half of its original value[8], given by the following equation:

$$t_{1/2} = \frac{ln2}{\lambda} \tag{2.5}$$

2.2.2 Ionizing Radiation

Ionising radiation is usually characterized by its ability to ionize an atom/molecule [9]. To ionize an atom (to cause valence electrons of the atom/molecule to escape to free space), 4 -25

eV is required[9]. The energy range encountered in applications of ionising radiation most frequently extends from 10 keV to 10 MeV but electrons and photons down to 1 keV have also experimental interest[9].

Ionising radiation is divided into two groups due to the way it interacts with matter:

1. *Direct ionising radiation*: Charged particles (electrons, charged nuclei) deliver their energies to matter directly through Coulomb –force interactions along the particle's track.

2. *Indirectly ionising radiation*: Uncharged particles (photons, neutrons) interact first with matter and deliver their energies to charged particles, later these charged particles *directly* interact with matter.

There are two types of photon radiation, x-rays, and γ- rays, which differ from each other by the way they are produced. γ-rays are produced through radioactive decay of an element which sends out a photon in γ energy range.

X-rays are generated in two different ways:

- Electrons interact with a target molecule and ionize the molecule by causing an electron to go to free space. The resulting empty place is filled by a higher energy level electron and the energy difference between these two levels is transferred to a photon. This kind of radiation is called *characteristic x-ray radiation* because the energy given to the photon depends on the target molecule.

- When an electron, which has negative charge, is driven onto a charged nucleus, the electron will be deflected by the electric field of the nucleus. This causes the electron to lose kinetic energy which is converted to a photon. By this way, the electron loses its energy. This is called *bremsstrahlung*, that is, *braking radiation*.

2.2.3 Interaction of Radiation with Matter

X- and γ- ray photons interact with matter by five types of interactions[9].

- Compton effect
- Photoelectric effect
- Pair production
- Rayleigh (coherent) scattering
- Photonuclear interactions

Only the first three of these are important because photons transfer their energies to electrons (in matter) which later lose the energies by Coulomb-force interactions along their tracks. On the other hand, photons in Rayleigh scattering lose/transfer no energy due to elastic scattering. Photonuclear interactions become significant only for photon energies above a few MeV which can lead to production of neutrons that could create radiation- protection problems.

During *Compton effect*, the incoming photon interacts with an *unbound* and *stationary* electron and transfers part of its energy to the electron which is assumed to be unbound and

stationary due to the large difference between the photon energy and the binding energy of the electron. The photon continues to interact with other electrons on its track which each time causes the photon to lose energy and change direction.

Photoelectric effect takes place when a photon hits a bound orbital electron in an atom/molecule where the photon vanishes by transferring its all energy to the electron. Pair production occurs if a photon energy is minimum 1,022 MeV [9]. The photon transfers all its energy to an electron- positron pair.

The first three important processes depend on the atomic number of irradiated element, *Z*, as follows[10]:

- Photoelectric effect $\sim Z^4$
- Compton effect $\sim Z$
- Pair Production $\sim Z^2$

This dependence can be seen in Figure 2.6 where one can see clearly that photoelectric effect is dominant for low photon energies and high Z, like lead.

When Z is low, such as in carbon and biological molecules, the dominant interaction process is Compton effect in the energy range 50 keV to 20 MeV.

As the photon energy increases, pair production becomes dominant.

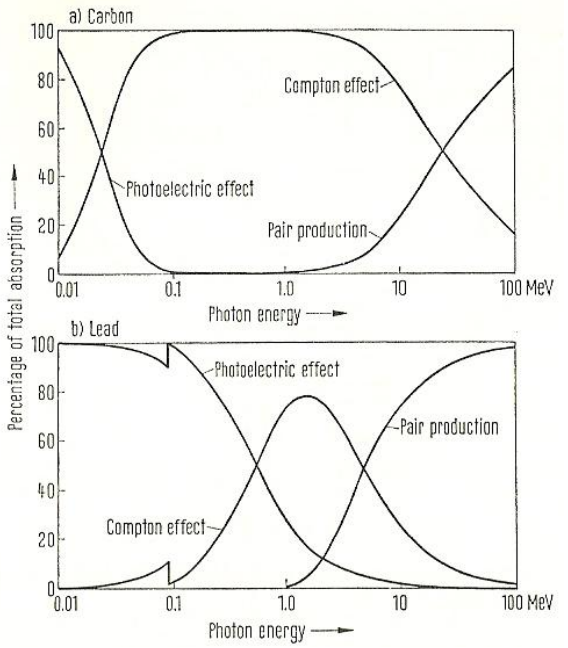


Figure 2.6: *Relative frequency of photoelectric effect, Compton effect, and pair production in carbon and lead[10].*

The electrons generated by these processes interact directly with target molecules losing their kinetic energies gradually in a process which is termed as *continuous slowing-down approximation* (CSDA). The probability for a charged particle not to interact with a layer of matter is zero and a 1 MeV charged particle would perform almost 10^5 interactions before losing its all kinetic energy[9]. These interactions are characterized by two parameters:

- *b* (impact parameter) = the distance of the charged particle when it passes an atom
- *a* = the radius of the atom

When ***b*** **>> a**, *soft collisions* take place where the particle's Coulomb-force field affects the atom as a whole leading to either excitation of the atom or ionization by ejecting a valence electron usually with a few eV. Since this type of collision is very probable, roughly half of the energy transferred to the absorbing medium is due to soft collisions.

When ***b*** **~ a**, *hard (knock-on) collisions* occur where the charged particle collides with an electron in the target atom, ejecting the electron (which is called δ *–electrons*) from the atom with a considerable kinetic energy. Whenever an inner-shell electron is ejected from an atom by a hard colision, characteristic x-ray photons and/or *Auger electrons* (which are freed by characteric x-ray photons) will be emitted just as if the same electron had been removed by a photon interaction[9].

When ***b*** **<< a,** the charged particle will be deflected by the electric field of the target atom and *bremsstrahlung* will occur.

Stopping power (dT/dx) for a charged particle with a kinetic energy T in a given medium is defined as the expectation value of the rate of energy loss per unit path length *x* in the medium [9]. Stopping power expresses the average rate of energy loss by a charged particle in all hard and soft collisions. The δ –rays which are produced by hard colissions can have enough energy to transfer kinetic energy a far distance away from the track of the primary particle and if one is measuring the dose in a small object, then the stopping power will overestimate the dose because the escaped δ –rays outside the object will be counted.

Therefore it is necessary to introduce a new , *restricted stopping power*, which is called **linear energy transfer** (LET) [9] which includes both soft collisions and those hard collisions resulting in δ –rays with energies less than a cutoff value Δ . LET is measured usually as keV /μm and it depends on radition energy. Electrons and photons are examples of low LET while protons and charged nuclei are examples of high LET as shown in Figures 2.7-8.

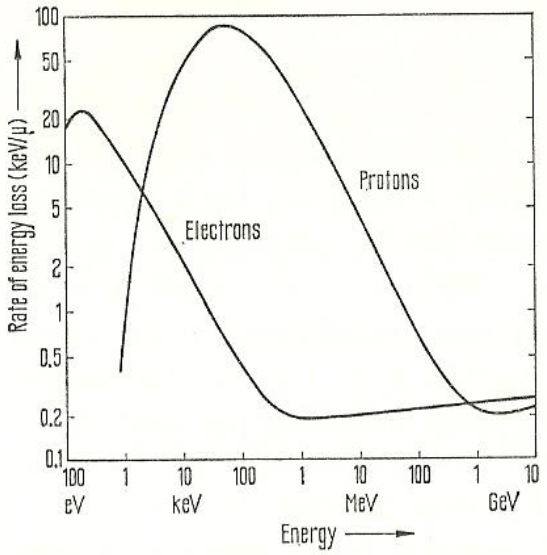


Figure 2.7: *Stopping power of electrons and protons in water, as a fuction of energy[10].*

Type of radiation	LET [keV/μ]
8 MeV γ-rays	0.2
^{60}Co γ-rays	0.3
200 keV X-rays	2.5
340 MeV protons	0.3
2 MeV protons	17
27 MeV α-particles	25
5 MeV α-particles	90
3.4 MeV α-particles	130
100 MeV carbon ions	160
160 MeV neon ions	450
330 MeV argon ions	1300

Figure 2.8: *Relative frequency of photoelectric effect, Compton effect, and pair production in carbon and lead[10].*

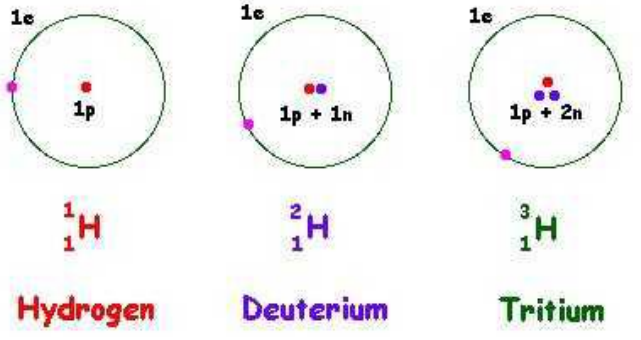
2.2.4 Tritium as Irradiation Source

Tritium is a radioactive hydrogen isotope. Its symbol is $[{}^{3}_{1}H]$ or T. It has one electron bound to a nucleus of two neutrons and one proton as shown in Figure 2.9.

It is unstable due to having an extra neutron in the nucleus which is why it undergoes β- decay which means converting one neutron into a proton, an electron and electron-type antineutrino:

$$n \rightarrow p + e^{-} + \bar{\nu}_e$$

Tritium decay is as follows:



$$\left[{}_1^3H\right] \rightarrow \left[{}_2^3He\right] + \beta^- + \overline{\nu_e}$$

where $\beta^- = e^-$.

Figure 2.9: *Illustration of Hydrogen, Deuterium and Tritium[7].*

The half-life of Tritium (this reaction) is 12.26 years [11]. The energy released in this reaction is shared by the electron and the antineutrino. Since antineutrinos have no charge and their *mean free path* in water is almost 1.7×10^{17} *m* [12], they do not interact with matter, i.e., with the cells. The electron average energy from Tritium's decay is 5.6 keV and the maximum energy is 18.6 keV. The corresponding mean free paths to these energies in *water* are 0.56 µm and 6 µm [11], respectively. This means even a piece of paper can stop these electrons. Due to both low energy and short mean free path, Tritium is used as a low –dose irradiation source.

2.2.5 Cobalt 60 (^{60}Co) as Irradiation Source

^{60}Co was used as source of radiation in some experiments. It has been made radioactive by bombarding stable ^{59}Co by neutrons. Its half-life is 5.26 years. It first undergoes β- decay resulting in maximum electron energy 0.32 MeV and later the recoiling nucleus, in two successive jumps, emits two photons one at 1.17 MeV and the other at 1.33 MeV and decays to ^{60}Ni as shown in Figure 2.10. The β- particles are absorbed in the cobalt metal and the stainless –steel capsules.

The γ- rays constitute the useful treatment beam.
These γ- ray photons lose their energies step by step by Compton effect[10] which is dominant process for biological molecules such as water.
LET value for ^{60}Co γ- ray is 0.3 keV /µm given in Figure 2.4.

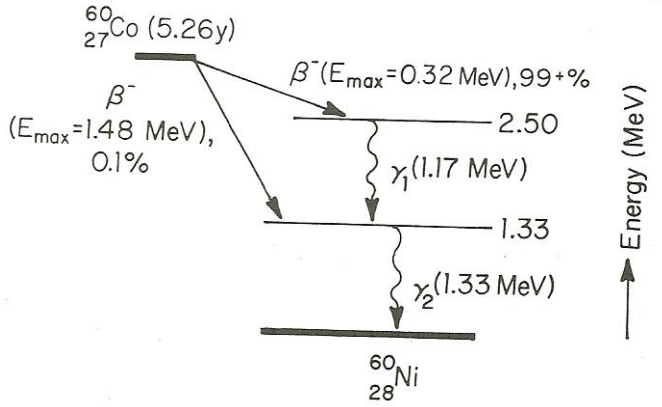


Figure 2.10: *Energy level diagram for the decay of ^{60}Co nucleus[13].*

2.3 Dosimetry

2.3.1 Absorbed Dose

The absorbed dose, *D*, is defined as the expectation value of the energy imparted to matter per unit mass *dm* at a point *P* in a volume *V*[9]:

$$D = \frac{d\varepsilon}{dm} \tag{2.6}$$

where $d\varepsilon$ is the expectation value of the energy imparted during some time interval in an infinitesimal volume *dv* at point *P*. Absorbed dose is measured in Gray (Gy) which is defined as 1 J/kg. The absorbed dose rate at a point P and time *t* is given by:

$$\frac{dD}{dt} = \frac{d}{dt}\left(\frac{d\varepsilon}{dm}\right) \tag{2.7}$$

2.3.2 Tritium Dosimetry

Tritium was attached to the protein valine added into medium which was later given to the cells. The dosimetry of this incorporated tritiated valine in T-47D human breast cancer cells was estimated by Åste Søvik[14] and by Ingunn Bjørhovde[15] and in T98G and T-47D human cancer cells were estimated by Ida Aspmodal[16]. All of them used the method which was developed by Goddu et. al.[17], where the cell was regarded as two concentric spheres both with density 1 g/cm^3 and with a homogeneous distribution of radioactivity inside cytoplasm and cell nucleus, respectively. Søvik, Bjørhovde, and Aspmodal found the following results:

Table2. 1: The average cell diameter and average cell nucleus diameter of T-47D and T98G cells.

Cell Type	Activity [Ci/mol] in the medium	Doserate to the cell nucleus [Gy/hour]	Average cell diameter [µm]	Average cell nucleus diameter [µm]
Søvik –T-47D	1.6	0.010 ± 0.001	19.00 ± 1	13.00 ± 1
Søvik –T-47D	2.5	0.041 ± 0.003	19.00 ± 1	13.00 ± 1
Bjørhovde -T-47D	1.6	0.015 ± 0.004	14.00 ± 1	11.00 ± 1
Aspmodal -T98G	1.67	0.0525 ± 0.001	20.66 ± 0.622	11.11 ± 0.182
Aspmodal–T-47D	1.67	0.060 ± 0.05	14 ± 1	11 ± 1

DNA in the cell nucleus is considered as critical target for radiation. The method assumes that the external radiation contribution to the cell is considered to be negligible, the energy transported outside the target area by δ-electrons was considered to be less important for low dose-rate electrons. The model does not give the dose-rate imparted to a cell with a particular size, cell geometry, and intracellular activity but the method estimates only the average dose-rate to a cell population. Since a lot of decays are necessary to give a significant dose when using low LET emitters (like Tritium that LET of Tritium electrons is 2-10 keV/µm[18]), this average dose-rate is considered to be relevant when describing the radio biological response of a cell population.

2.4 Cellular Radio Biology

2.4.1 Radiation Damage Repair and its Effects on Cell Cycle

Radiation damage of mammalians cells is generally divided into three main groups[1]:

- Lethal damage: It is irreversible and irreparable, and leads to cell death.
- Potentially lethal damage: This kind of damage leads to cell death under normal conditions but if the postirradiation environmental conditions are suboptimal for cell growth, then this is considered to be repaired.
- Sublethal damage: This can be repaired in hours unless additional sublethal damage is added, in which case both together can form lethal damage.

Mitotic death due to radiation is considered to be the most common form of cell death where cells die trying to divide themselves. The death can happen during first or subsequent division following irradiation. Necrosis is a process where the cells swell and burst.

Another way of cell death is termed *apoptosis* (a Greek word means "falling off") also known *programmed cell death*, is characterized by a stereotyped sequence of morphologic events[1]. As the cell dies, it shrinks (loses its water) and it separates into several membrane-bound fragments of different sizes called *apoptotic bodies*. Apoptosis after radiation seems usually to be a p53-dependent process where Bcl-2 is a suppressor of apoptosis[1].

The cells, which have lethal damage, die and they cause no cancer risk. There is cancer risk when the repair processes are active which may lead to mis-reparation and thereby, inactivation of suppressor genes or activation of oncogenes.

A radiation dose of 1 Gy in almost every cell nucleus generates roughly 2.10^5 ionizations, leading to around 1000 single-strand breaks in DNA, and 40 double-strand breaks in DNA but most of the cells survive due to effective repair processes[3]. Double –strand breaks in DNA (DSBs) are produced by radiation either *directly* or *indirectly*.

During *direct* radiation, the atoms in the DNA molecule may be ionized or excited whereas *indirect* radiation may especially interact with water molecules in the cell to produce free radicals like *Hydroxyl radical ($OH^{\bullet}$)* which can diffuse far enough to damage DNA molecules. Since 80% of a cell is composed of water[1], plenty of $OH^{\bullet}$ can be produced inside the cell by indirect action. This process is shown below where a water molecule is ionized, losing its 1 electron, later the ionized molecule interact with a water molecule which leads to an $OH^{\bullet}$.

A **free radical** contains an unpaired electron in the outer shell, making it highly reactive[1].

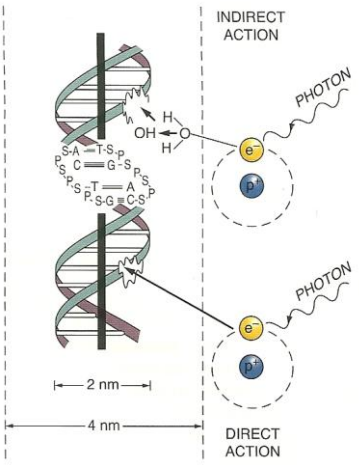


Figure 2.11: *Illustration of direct and indirect radiation on a DNA molecule[1].*

It is estimated that 2/3 of the damage to DNA by x- rays is caused by $OH^{\bullet}$ [1].

$$H_2O \rightarrow H_2O^+ + e^-$$

$$H_2O^+ + H_2O \rightarrow H_3O^+ + OH^{\cdot}$$

In both cases, the radiation leads to a biologic change in the DNA molecule either directly or indirectly. A DNA double – strand break (DSB) occurs if the breaks in the two strands are opposite one another or separated by only a few base pairs.

In eukaryotic cells, DNA DSBs are repaired by two fundamental processes[1]:

- Homologous Recombination Repair (HRR)

This requires an undamaged DNA strand which can be used as a template for the reparation. This is an error – free process because undamaged homologous chromatid/chromosome is used for the reparation.

- Nonhomologous End – Joining (NHEJ)

This is not an error-free process due to having no sister chromatid/chromosome (primarily found in G_1 phase of the cell cycle) to be used as template for the reparation. This may lead the cell to lose some of the genes which code for very important proteins for the regulation of the cell cycle such as p53, and p21. Such a cell without the gene of p21 can duplicate itself without control and lead to cancer.

As cells pass through the cell cycle, their radiosensitivity varies as demonstrated in Figure 2.12. Usually cells are most radiosensitive in G_2 and M. They are more resistant in the latter part of S-phase.

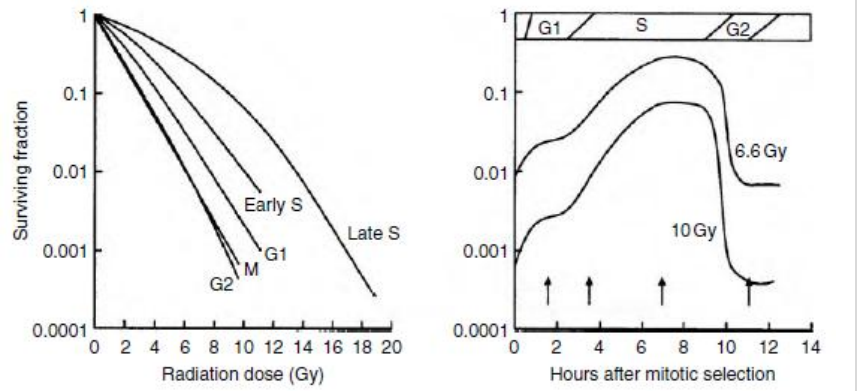


Figure 2.12: *The variation of radiosensitivity with the phase of the cells in the cell cycle [3].*

The reason why cells are so radiosensitive in G_2 is that the cells have little time to repair their damage before being called upon to divide. The curve of *Late S* is less steep compared to the curves of the other phases and it has a very broad shoulder due to effective reparation processes. On the right side of the figure one can see how different doses affect the surviving fraction hours after mitotic selection.

2.4.2 Dose Survival Measurements

A surviving cell is defined as a cell able to generate a colony of 40-50 cells, 5-6 generations[19]. The model which describes the relationship between the surviving fraction and the dose is called *Linear Quadratic* (LQ) model. This model is mathematically written as:

$$S = e^{-(\alpha d + \beta d^2)} \tag{2.8}$$

where *S* is the surviving fraction, *d* the dose and α and β are the parameters describing the linear and quadratic component, respectively, of the intrinsic radiosensitivity[20]. This model shortly says that the surviving fraction of cells is linearly proportional to the dose at low doses and it is proportional to the square of the dose at high doses[1]. Therefore the shape of the curve between the surviving fraction and dose in the beginning seems linear and later at high doses the curve starts to bend. This can be seen in Figure 2.13. Two different human hepatocellular carcinoma (HCC) HepG2 and Hep3B cell lines were used and their responds were also different.

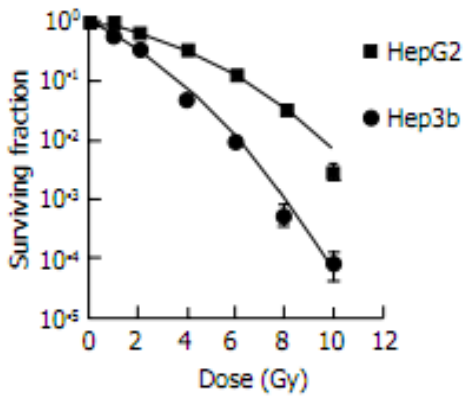


Figure 2.13: *Standard dose-survival curves of HepG2 and Hep3b fitted with standard two parameter LQ model[21].*

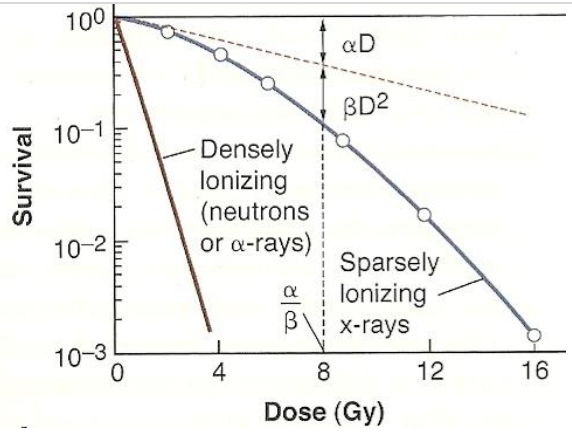


Figure 2.14: *Shape of survival curve for mammalian cells exposed to radiation. For high LET (densely ionizing) particles the curve has a straight line and for low LET (sparsely ionizing) particles it has an initial slope followed by a shoulder which is later straighten out for high doses[1].*

The effect of low and high LET particles on the survival curves is demonstrated in Figure 2.14. When the linear and the quadratic cell killing components are equal to each other, $\alpha D=\beta D^2$, the ratio $\alpha/\beta=D$, is the dose which determines the size and the shape of the shoulder of the survival curve. The size of the shoulder reflects the amount of repair of sublethal damage. Late-responding tissues are characterized by a broad shoulder corresponding to a small α/β ratio which indicates a lot of sublethal repair as illustrated in Figure 2.15. On the other hand, tumour and early responding tissues have a large α/β ratio due to higher linear component (α) effect at low doses. When there are higher doses, the LQ model predicts a continuously bending which is not agreeing with experimental results, but in the range of clinical doses in radiotherapy, this prediction seems to be adequate.

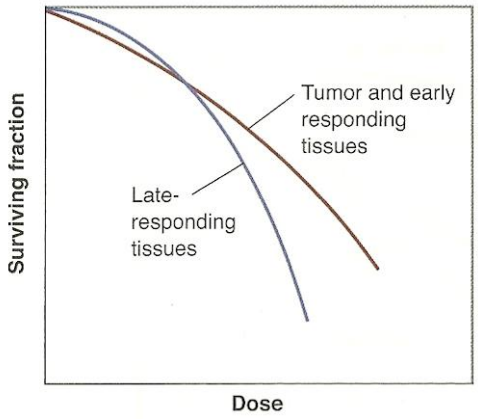


Figure 2.15: *Survival curves for late and early responding tissues[1].*

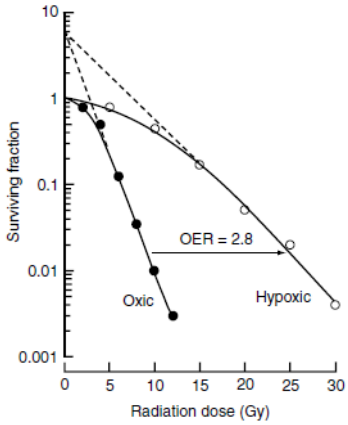


Figure 2.16: *Survival curves for cultured mammalian cells exposed to x-rays under oxic or hypoxic conditions[3].*

The survival curve of tumour/normal tissue can be altered by *oxygen effect* as seen in Figure 2.16. The **oxygen enhancement ratio** (OER) is defined as the ratio of doses applied under hypoxic to aerated conditions required to achieve the same biological effect[1]. For low LET particles, OER is between 2.5 and 3.5. A tumour tissue contains a mixture of aerated and hypoxic cells. If one irradiates the tumour tissue with a dose of x-rays, most of the aerated cells can be killed since they are more radiosensitive. Right after the irradiation, most of the cells in the tumour will be hypoxic which can be later re-oxygenized. The new aerated cells can be killed in the same way until the presence of hypoxic cells does not have a significant effect on the outcome of the multi-fraction regimen[1].

2.4.3 Low Dose Rate

It is possible to change the biologic effect of a given dose by altering the dose-rate. The effect obtained by this process is called the dose –rate effect. When the dose-rate is reduced while increasing the exposure time, the biologic effect is generally reduced due to giving enough time to the cells to repair their sub-lethal damages[1].

The total dose D is delivered in several equal fractions (D1, D2, D3, D4 and so on) where each fraction causes a shoulder resulting a sparing effect. If one assumes that the number of these small fractioned low doses is infinite then the curve generated from sum of the all these small doses will be a straight line with no shoulder. The dose-rate effect which is caused by the reparation of sub-lethal damage changes dramatically between 0.01 and 1 Gy/min[1].

Figure 2.17 summarizes the effect on surviving fractions using high and low dose-rates. An acute high dose –rate will lead to a steep curve with an initial large shoulder. As the dose-rate decreases and exposure time increases, the curve gets shallower while the

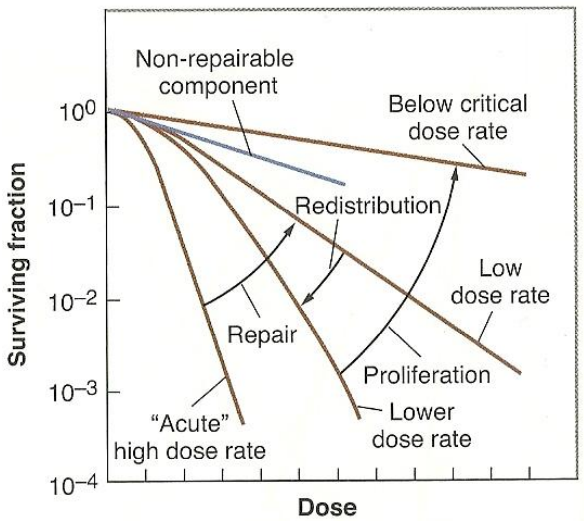


Figure 2.17: *The dose rate-effect. As a result of sub-lethal damage repair, the dose –response curve gets shallower when the dose-rate is reduced[1].*

shoulder becomes smaller due to reparation of sub-lethal damages. If one decreases the dose-rate more, in some cell types such as HeLa cell line, the efficiency of cell killing increases. This effect is called *inverse dose–rate effect*. Why this occurs is explained like this: The low dose-rate (LDR) causes the cells to continue through the cell cycle and later accumulate in G_2, which is the most radiosensitive phase of cell cycle. If the dose-rate is low enough for the cells to pass G_2 and divide themselves, proliferation effect is observed which increases the number of cells. Another explanation to the inverse dose-rate is that the reparation processes are not performed with the low dose-rates. That is why there is inverse dose-rate effect in some cells which shows hyper radiosensitivity (HRS) with acute low doses[22].

2.4.4 Hyper Radiosensitivity

When one looks at Figure 2.18(B), something seems strange at low doses up to 2 Gy. Figure 2.18(A) shows that that area was supposed to be linear (dashed line) according to the LQ model but it is not and it occurs because of some cells which are low-dose hyper-radiosensitive. This means it is possible to kill or inactivate many cells by using a low-dose.

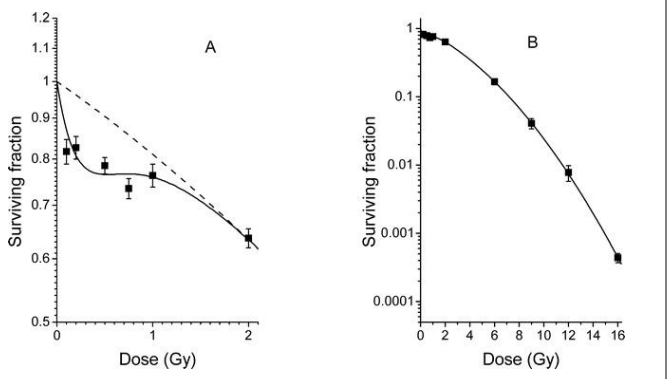


Figure 2.18: *T-47D cells irradiated with a single acute dose of ^{60}Co γ radiation. Data points represent means values from 12 independent experiments. The curves represent fits to the data of the IR model (solid line) and the LQ model (dashed line)[23].*

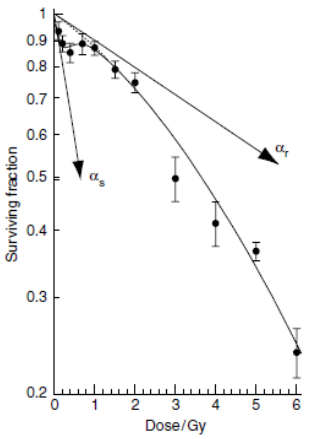


Figure 2.19: *The dose rate-effect. As a result of sub-lethal damage repair, the dose – response curve gets shallower when the dose-rate is reduced[1].*

Low-dose hyper-radiosensitivity (HRS) has been detected in many cells in vitro to doses of ionizing radiation lower than ~0.5 Gy[23]. As the dose is increased above about 0.3 Gy, repair processes contribute to increased radioresistance (IRR)[23-24]. The solid line in Figure 2.18(A) is described by the *induced repair-model* (IR-model) by modifying the α in equation (2.8) :

$$\alpha = \alpha_r \left\{ 1 + \left(\frac{\alpha_s}{\alpha_r} - 1 \right) e^{\frac{-d}{d_c}} \right\} \tag{2.9}$$

where d is dose, α_r is the value of α extrapolated from the high dose LQ response (equation 1), and α_s is the actual value of α derived from the initial part of the curve (at very low doses). d_c is the dose where the change from α_s to α_r is 63% complete[8]. One can clearly see in Figure 2.19 that α_s is slope of the IR-model where α_r is the initial slope of the LQ-model.

Howard and Pelc showed that cells arrest cell cycle progression after low-dose radiation damage not in S or M but in either G_1 or G_2. Later they noticed that primary cells would arrest both in G_1 or G_2, but tumor cells often would show only the G_2 arrest point.

A second early and transient (active 0-2 hour postirradiation) G_2/M checkpoint, at which cells irradiated in G_2 are arrested, was discovered by Xu et al.[25]. The checkpoint depends on ATM activation and remains inactive at doses less than almost 0.4 Gy, the same dose range in which HRS occurs which is why it has been assumed that HRS reflects failure to arrest damaged G_2-phase cells for repair before entering mitosis[23].

It is known that HRS can be eliminated or reduced temporarily by pre-exposure of the cells to chemicals or small radiation doses (a priming dose) in vitro. Edin et al, showed that a priming dose of 0.3 Gy γ rays eliminated HRS in T-47D breast cells when given both as acute irradiation (40 Gy/h) and as low-dose-rate irradiation (0.3 Gy/h). The priming effect lasted for less than *24 hours* after an acute dose priming irradiation (40 Gy/h), while the same priming dose of 0.3 Gy given at a dose rate of 0.3 Gy/h showed to induce a non-hyper-radiosensitive phenotype of the T-47D cells that continued for *several weeks*[23]. This is consistent with a previous study performed by Edin et al.[26].

It is also reported that in certain cell cells radiation causes cells to generate signals released into the environment that can cause cytotoxic effects in unirradiated cells exposed to the irradiated-cell conditioned medium(ICCM)[23]. Medium transferred from the low-dose-rate primed cells to unirradiated cells eliminated HRS in recipient cells even if the donor cells had been cultivated for 14 months after the priming dose. When the unirradiated cells were cultivated with fresh medium for two weeks, the cells regained HRS[23]. To sum up, LDR priming of T-47D cells in addition to LDR priming of medium conditioned on T-47D cells induce a factor in the medium which cause the early G_2-checkpoint to be activated in recipient cells by doses usually in the HRS dose range[20].

2.5 Flow cytometry

2.5.1 Introduction

Flow cytometry is described as a system for sensing cells or particles as they move in a liquid stream through a laser beam past a sensing area[27]. The applications of flow cytometry have spread through all branches of biological sciences. The importance of flow cytometry can be also estimated from the increasing reference to "flow cytometry" in the Medline –indexed literature over the past three decades shown in Figure 2.20[28].

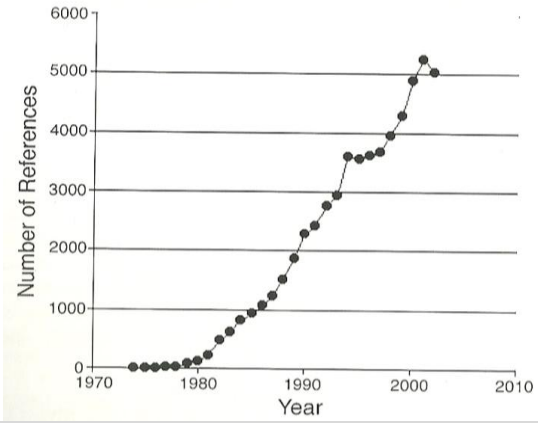


Figure 2.20: *Increasing reference to "flow cytometry" in the Medline-indexed literature over the past three decades. The actual use of flow cytometers predates the use of the term itself[28].*

According to all developments in the history of flow cytometry (see *Appendix C*), flow cytometry highly depends on advances in other scientific areas such as fluid dynamic, electronics, microscopy, optics, lasers, computer, antibody technology, in jet technology.

2.5.2 Instrumentation (Components of a flow cytometer)

The flow cytometer system that was used during this master project is shown in Figure 2.21. It has a computer system and a white-red colour machine surrounded with some bottles, *BD Accuri C6* flow cytometer. The flow cytometer has two lasers (488nm and 640nm) as excitation source, 4 colours and user-swappable optical filters as emission detection, maximum 10,000 events (cells/particles) per second as data acquisition rate and many other specifications[29].

Figure 2.21: *BD Accuri® C6 Flow Cytometer [29].*

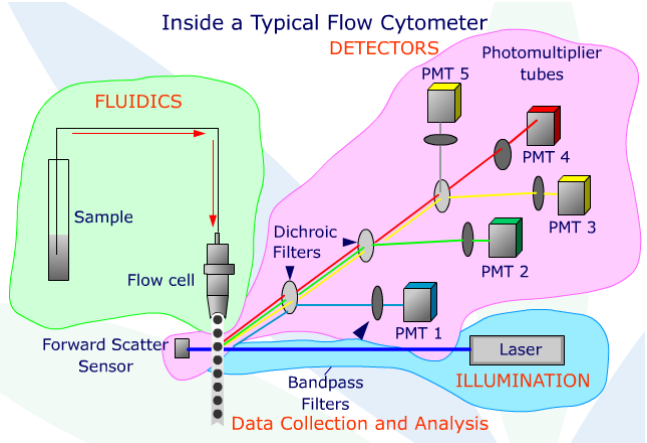


Figure 2.22: *Inside a typical flow cytometer, illustrated by Dr. J. Paul Robinson, Purdue University Cytometry Laboratories[30].*

The components of a typical flow cytometer system are divided into three parts:

- Illumination system
- Fluidic system
- Detector system

The detector system is combination of both optical and electronic systems.

2.5.2.1 Illumination system

Illumination sources usually are [28 and 31]:

- Lamps
  - Arc Lamps
  - Qartz-Halogen Lamps
- Lasers

	Emission wavelengths (nm)
Argon ion	351, 364, 488, 514
Red helium-neon (He-Ne)	632
Green helium-neon (He-Ne)	543
Red diode	635
Krypton ion	568, 647

- Light Emitting Diodes (LEDs)

Lamps were used before and they are not common any more. These are broadband light sources which produce white light which has peaks across the spectrum. Therefore this needs optical filtering to select the desired excitation wavelengths.

In contrast, lasers (Light Amplification by Stimulated Emission of Radiation) provide one or a few well-defined peaks allowing more selected illumination of the sample. The physical process behind all lasers is *stimulated* or *induced emission*, described by Einstein in the early 1900's. The advantage of a laser beam is that the photons are monochromatic and coherent[31]. As to LEDs, they have gained popularity due to their selected wave lengths, low energy consumption, low cost and long life time.

But most of the flow cytometers today (like *BD Accuri C6*) use lasers as the illumination source. A laser beam has a circular, radically symmetrical cross-sectional profile, with a diameter of approximately 1-2 mm. Lenses in the cytometer itself are used to shape the laser beam and to focus it to a smaller diameter as it illuminates the cells[32]. The important thing is that the narrower the beam is, the more quickly will a cell pass through it- giving opportunity for the signal from that cell to drop off before the start of the signal from the next cell in line and avoiding the coincidence of two cells in the beam simultaneously.

2.5.2.2 Fluidic system

The BD Accuri C6 flow cytometer has four bottles. They are *waste bottle, cleaning solution bottle* and *decontamination bottle*. Other components of this system are *sample tube*, a *pump*, and a *flow cell (flow chamber)*. Figure 2.23 illustrates how the sample and sheath fluids are carried into the flow cell. The sheath fluid reservoir is pressurized, usually with pumped air, to drive the sheath fluid through a filter to remove extraneous particles and then through plastic tubing to the illumination point (interrogating point, analysis point). The sheath stream is usually composition of phosphate –buffered saline solution for mammalian cells[32] but water was used as sheath fluid in this project.

Figure 2.24 shows what happens if there is low pressure difference between sample and sheath fluid. This means the sample fluid is injected slowly and the cells flow one at a time through the center of the laser beam. This is seen on the left flow cell in the figure. If the pressure difference is high, as shown in the right flow cell in the figure, the sample flow rate will be high which means more than one cell will pass through laser beam at the same time. This is not desirable because it leads to bad data.

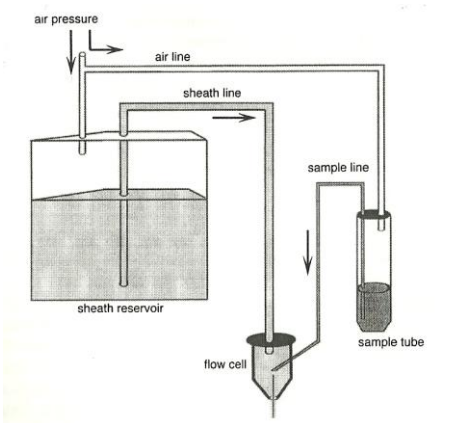


Figure 2.23: *The fluidic system, with air pressure pushing both the sample (with suspended cells) and the sheath fluid into the flow cell[32].*

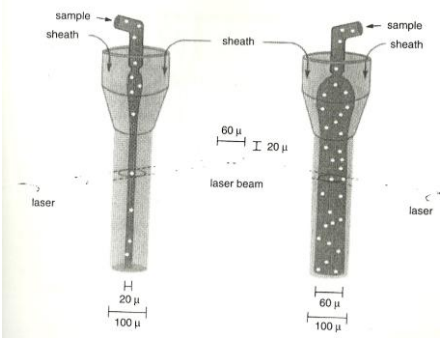


Figure 2.24: *The flow cells within the core of sheath fluid through the analysis point in the illumination system[32].*

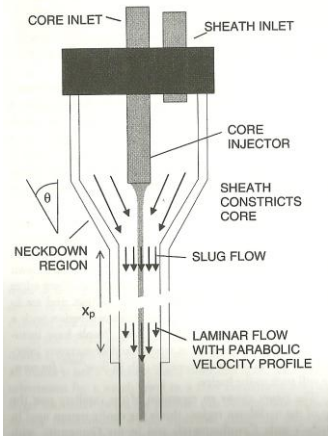


Figure 2.25: *The fluidic system, with air pressure pushing both the sample (with suspended cells) and the sheath fluid into the flow cell[31].*

Since a flow cell is considered to be the most important part of a flow cytometer, it is better to focus on it little bit more. Figure 2.25 shows more details about a flow cell. Due to *the law of conservation of mass*, which is given by equation (2.10) and also called *the continuity equation*[33], the flow velocities at different points in the system will be different, i.e., higher in the narrow portions than in the wider ones[31]. The introduction of a large volume into a small volume in such a way that it becomes "focused" along an axis is called *hydrodynamic focusing*.

$$\frac{\partial_\rho}{\partial_t} + \nabla.(\rho \boldsymbol{u}) = 0 \qquad (2.10)$$

where $\mathbf{u} = \mathbf{u}(\mathbf{r},t)$ is the velocity of the flow, $\rho = \rho(\mathbf{r},t)$ is the density of the flow and $\mathbf{r}$ is the position vector.

If one considers a cylindrical tube of radius *R* containing flowing water, one can see that the velocity of water at different distances from the axis or center of the tube varies. It is highest along the axis; at the walls of the tube, there is a thin *boundary layer* of water that is not moving, that is, its velocity is zero. At any intermediate point a distance *r* along the radius, the velocity is proportional to $(R\text{-}r)^2$. This creates a *parabolic profile* of flow velocities, as if the water were broken up into thin cylindrical layers (*laminae* in Latin) sliding over one another[31]. Flows are divided into two groups, *laminar flows* and *turbulent flows*. Laminar flows are rather smooth with fluid (gas) gliding in well-defined layers. Motion of any fluid parcel in the laminar flow may be predicted if the velocity field is known. On the contrary, motion of a parcel is absolutely unpredictable in a turbulent flow, since it involves chaotic pulsations of velocity and pressure.

In Figure 2.25 it is seen that both sheath and sample fluids are injected into the flow cell. After a point near the core (sample) injector tip, the cross section of the flow cell is gradually decreased; the length of the flow cell over which this happens is called "*neckdown region*". At this point it is important to avoid anything in neckdown region which can generate turbulence. Sharp edges and/or sudden changes in diameter can do that. A neckdown region with a gentle conical taper ($\Theta \approx 30^0$) is considered to be good to avoid turbulence[31]. It is useful to mention that the differences in diameter are usually between 10- and 40 fold, bring about an increase in velocity equal to 100- to 1600-fold[28]. After the neckdown region, the velocity profile at the entrance to the constricted region is nearly constant across almost the entire cross section. This is known as "*slug flow*". If the fluid flows for some distance, X_P, through the constricted portion of the tube, parabolic laminar flow profile will be re-established. X_P is given in equation (2.11).

$$X_p = 6\ x\ 10^{-5}\ d^2\ v \tag{2.11}$$

where d, in µm, is the diameter of the constricted portion of the tube and v, in m/s, is the average of the fluid velocity[31]. This equation can be useful when one wants to design a flow cell because it can be estimated at which distance the cells should be illuminated so that the cells flow one at a time through the centre of the laser beam.

2.5.2.3 Detector system

Detector system can be divided into two systems: *Optics* and *Electronics*. At this point, one can suppose that the cells are illuminated and now it is time to collect light (information) from them. The light which will be collected comes from fluorochrome (also known as *fluorophores*) molecules which are labelled to an antibody that binds itself to a specific molecule on the cell surface or inside the cell. Since they become part of the cell, it is assumed that the light comes from the cell.

2.5.2.3.1 Optics

In Figure 2.11, two types of optical filters are shown which are *band-pass* and *dichroic filters*. Optical filters are divided to four groups[30]:

➢ Long pass filters	Transmit wavelengths *above* a specific wavelength and the rest is reflected.
➢ Short pass filters	Transmit wavelengths *below* a specific wavelength and the rest is reflected.

➢ Band pass filters	Transmit wavelengths in a narrow *range* around a specific wavelength. Band width can be specified between 1 nm and 25 nm. They are usually used immediately in front of the detector as shown in Figure 2.22.
➢ Neutral density filters	Reduce intensities without changing the wavelengths.

So as to dichroic filters/mirrors (sometimes called *beam splitters*[34]), they can be long or short pass filters. When they are placed at a 45^0 angle to the incoming beam (as shown in Figure 2. 26) some light can be transmitted by the filter depending upon its properties but some light is reflected at a 90^0 angle (called *mirror* because of its reflection).

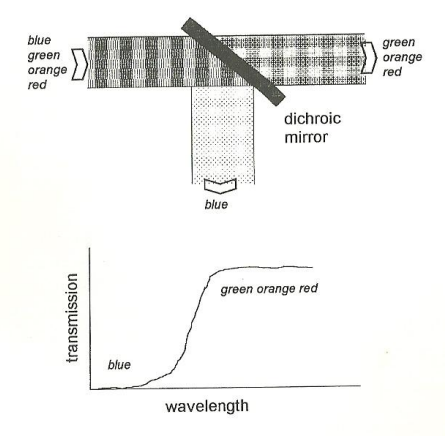


Figure 2.26: *An illustration of the properties of a long pass dichroic filter [34].*

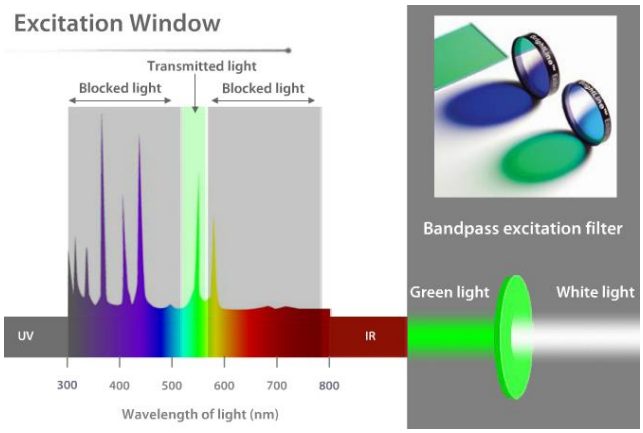


Figure 2.27: *An illustration how band pass filter is used to select green excitation window when the illumination source is white light[35].*

If the illumination source is a lamp, which produces white light, then one will get many intense different excitation peaks across the spectrum and in order to select the desirable excitation peak, optical filtering is needed. This is well visualized in Figure 2.27 where green light is chosen as the selected excitation peak. In addition to this, later an extra band pass emission filter can be used right in front of the detectors. The light beam is separated into specific wavelengths by the help of the filters, and mirrors. Now they are ready to hit the detectors.

2.5.2.3.2 Electronics

There are two broad classes of optical detectors: *photon detectors* and *thermal detectors*[36].

➢ <u>Photon detectors</u>

Photons produce free electrons. In order to perform this, they must have sufficient energy to exceed some threshold. Two common photon detectors are *photodiodes* and *photomultipliers (PMTs).*

➢ <u>Thermal detectors</u>

These respond to the heat delivered by light. Their respond is independent of wavelength as shown in Figure 2.28.

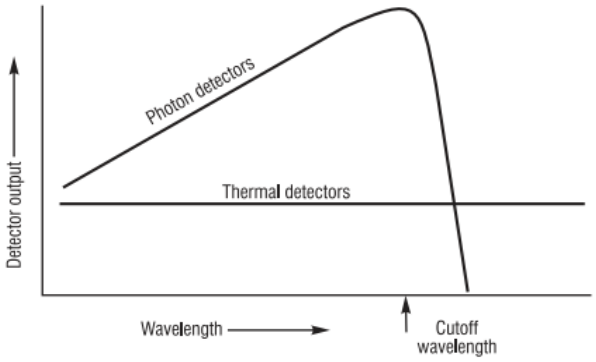


Figure 2.28: *Schematic drawing of the relative output per unit input for photon detectors and thermal detectors as a function of wavelength. The position of the long-wavelength cutoff for photon detectors is indicated[36].*

The detectors generally used are those best suited to the numbers of photons with which one will have to deal. It is common to use *photodiodes* as detectors for absorption, extinction, and forward scattered signals and *photomultiplier* as detectors for orthogonal scatter (side scatter) and fluorescence signals[15].

Forward scatter, side scatter and fluorescence signals can be seen in Figures 2.29(A-H). In the figures first a laser beam is coming onto a cell, eventually it hits the cell and scattered in all directions (in all angles), mostly in forward direction with small angles. Forward direction is shown clearly and it targets the detector. Side scatter directions on a cell and in the flow cytometer as well as the fluorescence detection are also shown.

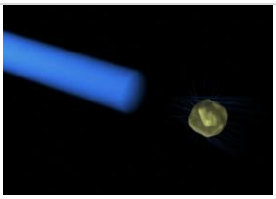


Figure 2.29(A): *A blue laser beam is coming onto a cell [35].*

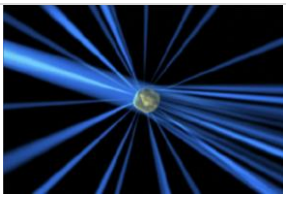


Figure 2.29(B): *Light is scatted in all directions, dominantly in forward direction [35].*

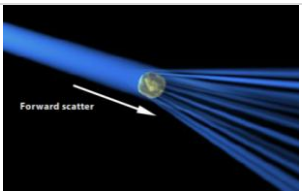


Figure2.29(C): *Forward scattering is illustrated [35].*

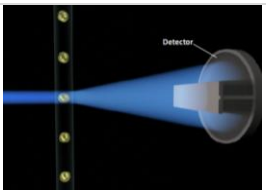


Figure 2.29(D): *Detection of forward scatter on a detector[35].*

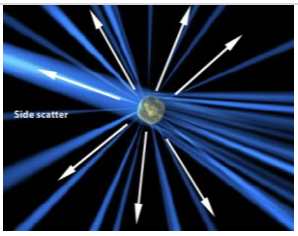


Figure 2.29(E): *Directions of side scatter is marked[35].*

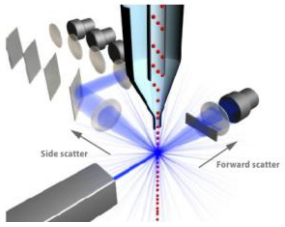


Figure 2.29(F): *Both side and forward scatters are shown in the flow cytometer [35].*

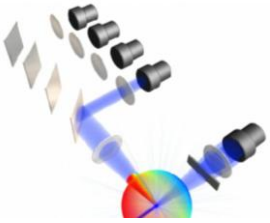


Figure 2.29(G): *Fluorescence detection starts[35].*

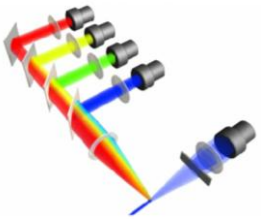


Figure 2.29(H): *Fluorescence detection ends[35].*

Since the beam is continuously directed onto the forward scatter detector, to prevent the detector from the damage a blocking/obscuration bar is used in front of the detector all the time. In addition to this, a neutral density filter is used to attenuate the intensity of the forward scattered light. This filter should be reflective rather than absorptive so it does not melt or catch fire [31]. When the light passes through the filter, in the end it will reach the detector which is a photodiode.

Photodiodes produce electrons from photons and these electrons are considered as current at their anodes. The gain, which is described by *quantum efficiency* (number of photons in divided by number of electrons out) times 100%), is usually one electron out per one photon in. That is why photodiodes are not sensitive [37]. Photodiodes are usually operated in the *photovoltaic* mode, in which no external voltage is applied to the diode; they can also be run in the *photoconductive* mode, with a *bias voltage* applied. Since photovoltaic and photoconductive photodiodes have no gain, the small currents which they produce in response to incident light must be amplified electronically. This is usually done by an active electronic preamplifier circuit that converts small input currents to proportional, but much larger, output voltages[31].

Like photodiodes, *photomultiplier tubes* produce current at their anodes when photons impinge upon their light sensitive *photocathodes*. On the other hand, PMTs require external power sources. Their gain can be quite high (10^7 electrons out for each photon in)[31]. An illustration how a photomultiplier is operated is demonstrated in Figure 2.30 (A). When photons hit the photocathode, it emits electrons. As shown in the figure, PMT contains a series of electrodes called *dynodes*, to each of which is applied a potential slightly more positive than that on its neighbour dynode nearer the photocathode.

The electrodes are arranged in space so that photoelectrons (emitted from the photocathode) are accelerated toward the first dynode by the electric field set up by the potential difference between these electrodes.

When they hit the next dynode, electrons leads to *secondary emission* of more electrons from the dynode surface; these are accelerated toward the next following dynode again by the electric field between the dynodes, and then produce more secondary emission, and so on. All these occur in *vacuum* that is why PMTs are known also as *vacuum tubes*.

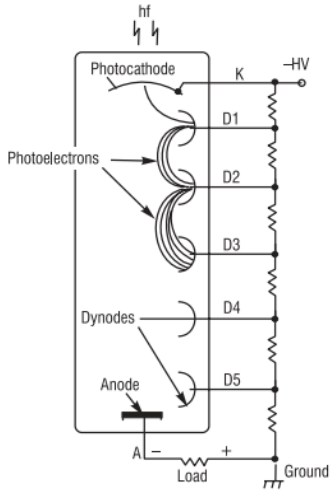


Figure 2.30(A): *Principles of photomultiplier operation. The dynodes are denoted D1, D2, etc. [36].*

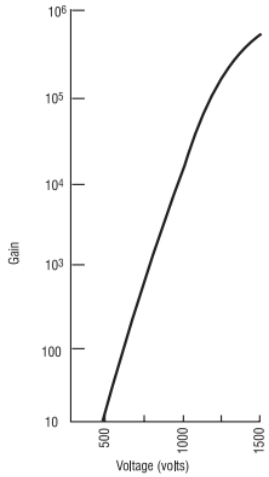


Figure 2.30(B): *Photomultiplier gain as a function of applied voltage [36].*

The higher the applied potential between the dynodes, the more energy is imparted to the electrons between stages. This causes emission of increasing numbers of secondary electrons from each successive dynode surface and increases the gain of the tube as shown in Figure 2.30(B)[31]. The spectral response of a PMT is determined by the composition of the photocathode which means one can determine to which spectral range the detector should be sensitive or not.

In many cytometers, the fluorescence (side scatter colour detectors) are simply given numbers (e.g., FL1, FL2, FL3)[32]. Figures 2.31(A and B) summarize what the electronic system does.

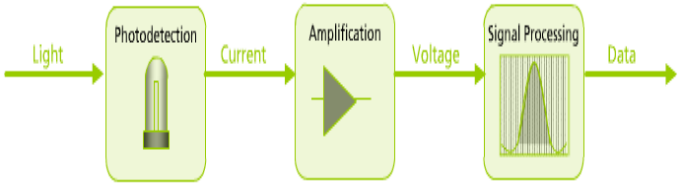


Figure 2.31(A): *Signal processing[38].*

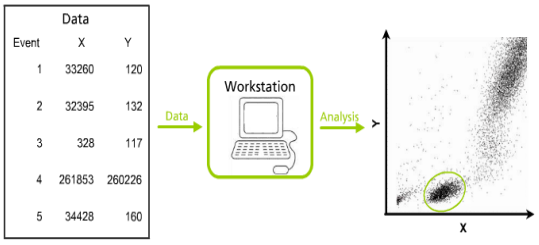
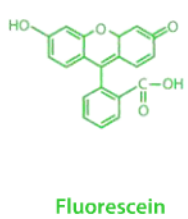


Figure 2.31(B): *Data storage and analysis[38].*

First light (photon) comes onto the detector which produces a lot of electrons (in case of a PMT). These electrons generates a current which is amplified either linearly (in case of no broad range of signals) or logarithmically (in case of broad range of signals) and converted into voltages (Remember an amplifier adjusts the intensity of the signal while keeping the wavelength constant.). These voltage signals are then digitalized by using binary system corresponding to their signal heights, widths, and areas. These data is stored as files in a computer. A data analysis program such as FCS Express can be used to graph and analyse the files.

2.5.3 Fluorochromes and Fluorescence

A *fluorophore* is a ring shaped molecule (shown in Figure 2.31(A) which is capable to absorb a specific range of light and get excited by this extra energy. During a very short range of time (from 10^{-15} to 10^{-9} seconds[35]), the excited electrons relax to the lower excited states by losing some of their extra energies which are in the form of heat. But this is not enough to be stable as they were in their ground states so after these very short ranges of times they eventually return back to their stable ground states by emitting light which is also known *fluorescence* shown in Figure 2.32(A).



Fluorescein

Figure 2.31(A): *Flourescein (FITC) as a fluorophore molecule[35].*

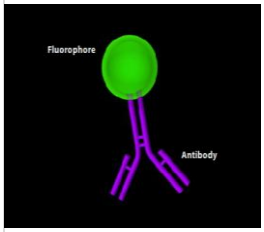


Figure 2.31(B): *A Fluorophore bound to an antibody, called fluorophore-labelled antibody[35].*

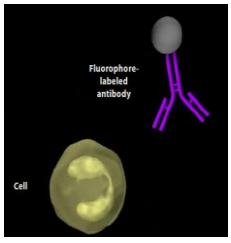


Figure 2.31(C): *A fluorophore-labelled antibody near a cell[35].*

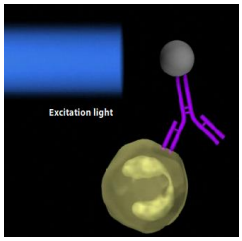


Figure 2.31(D): *Excitation light comes onto the fluorophore-labelled antibody which is bound to an antigen on the cell surface[35].*

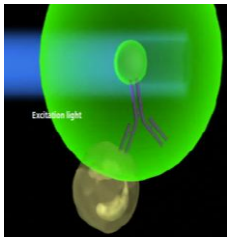


Figure 2.31(E): *The fluorophore first absorbs the light and then emit the light[35].*

As a result of this process, the emitted light will have a longer wavelength (less energy) than that of incoming absorbed lights therefore the emission peak will shift to right side of its absorption peak. This wavelength difference is called *Stokes shift* which is visualized in Figure 2.32(B). In this project *fluorescein (FITC*), and *propidium iodide (PI)* were used as fluorophores.

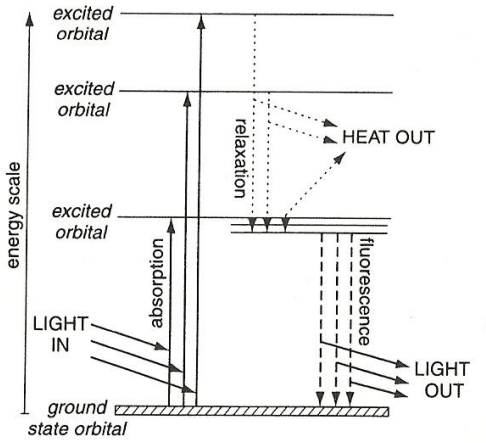


Figure 2.32(A): *Illustration of how fluorescence occurs[26].*

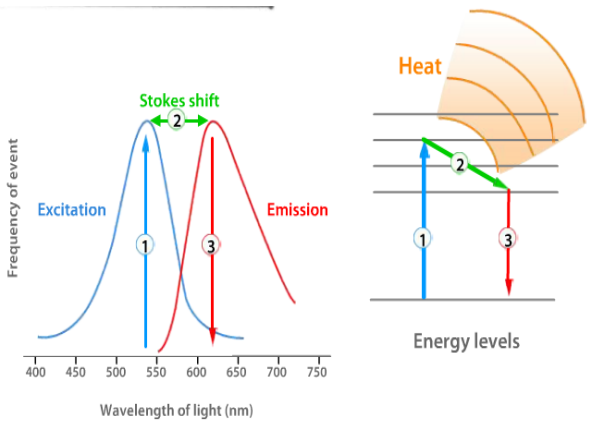


Figure 2.32(B): *Illustration of Stokes shift and representatives energy levels[35].*

An illustration of how a fluorophore emits light is shown in Figures 2.31 (B-E). One can see that the fluorophore is bound to an antibody which is later bound to an antigen on the cell surface. Therefore it is suitable to learn little bit more about antibodies.

Antibodies, or immunoglobulins, are proteins produced by the immune system in response to foreign molecules. Each antibody binds to a target molecule, called *antigen*, in order to either inactivate it or mark it for destruction.

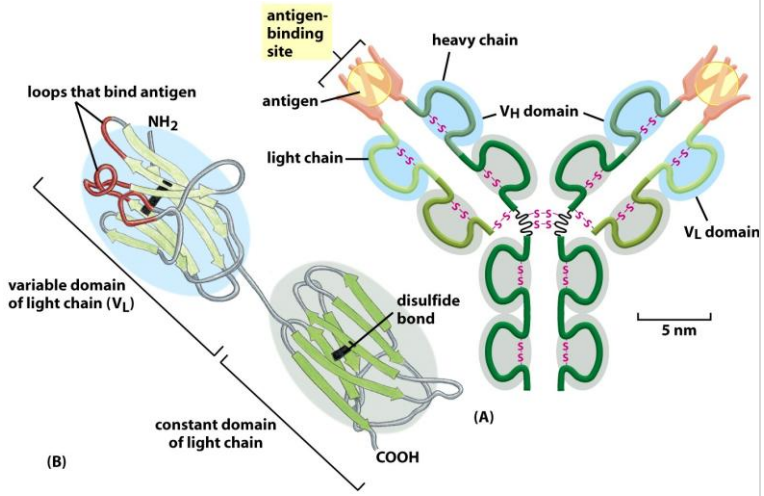


Figure 2.33(A and B): *This is figure 3-41 from Alberts' book. It illustrates an antibody molecule[2].*

A typical antibody is Y-shaped and has two identical binding sites for its antigen, one on each arm of the Y, as shown in Figure 2.33 (A). The protein is composed of four polypeptide chains (two identical heavy chains and two identical and smaller light chains) held together by disulphide bonds. Each chain is made up of several different immunoglobulin domains, here shaded either *blue* or *gray*. The antigen-binding site is formed where a heavy-chain variable domain (V_H) and a light-chain variable domain (V_L) come close together. These are domains that differ most in their sequence and structure in different antibodies. Each domain at the end of the two arms of the antibody molecule forms loops that bind to the antigen. One can see these finger-like loops (red) contributed by the V_L domain in Figure 2.33 (B).

2.5.4 Data Analysis

2.5.4.1 Histograms

There are two parameters which affect the signals obtained by *forward scatter*.

- One of them is the size of the cell/molecule, as shown in Figure 2.35(A). The larger cell size is, the more forward scatter occurs which leads to bigger voltage pulses in the computer system.
- Like in Snell's law, the incident and refracted angles and their refractive indexes affect each other. The larger the difference in refractive indexes of sheath fluid and the cell, the more forward scatter is generated.

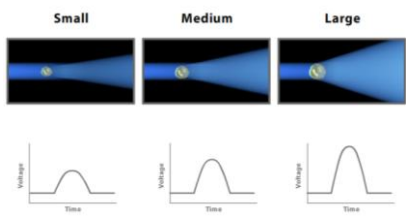


Figure 2.35(A): *The amount of forward scatter and correspondent voltage pulses according to cell size[35].*

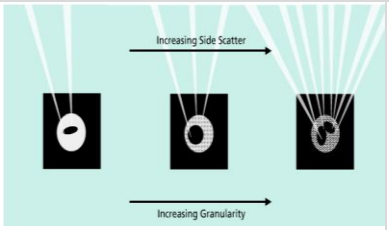


Figure 2.35(B): *The amount of side scatter and correspondent voltage pulses according to cell granularity[38].*

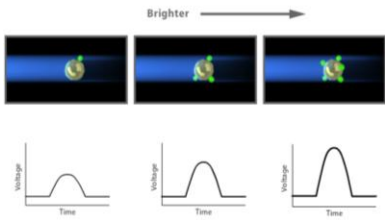


Figure 2.35(C): *The amount of fluorescence and correspondent voltage pulses [35].*

As to side scatter, it is based on the granularity or internal complexity of a particle/cell. As shown in Figure 2.35(B), the more granular the cell, the more side scatter is generated as the cell passes through a laser beam. This means, as in Figure 2.35(A), the higher voltage pulses will be created when granularity increases.

As to fluorescence, a flurophore-labelled antibody binds itself to an antigen either on the cell surface or inside the cell and there is not only one flurophore-labelled antibody or antigen per cell. There are thousands of them on a cell and in the sample solution. So some cells will bind themselves to more antibodies and some less and this will also generate a distribution. As shown in Figure 2.35(C), the more fluorescence we get from a cell, the brighter they will be which leads to higher voltage pulses.

A computer will convert the voltage signals to numbers called channel numbers. Later the intensities versus the channel numbers can be plotted in histograms.

In Figure 2.36 (A-C) three different types of cells with different sizes and internal complexities in a sample fluid are shown. These cells can be utilized to analyse the differences among forward scatter, side scatter and fluorescence.

Less internal complexity and size.

Figure 2.36(A): *Illustration of a lymphocyte[35].*

More internal complexity and the largest size.

Figure 2.36(B): *Illustration of a monoctye[35].*

The most internal complexity and the second largest size.

Figure 2.36(C): *Illustration of a neutrophil[35].*

What one sees in Figure 2.37(A) is the one dimensional forward scatter histogram. The three types of cells are distributed on the histogram according to their sizes. For example, the largest cells are on the right side of the histogram. This histogram alone does not tell us much about cellular parameters such as internal complexity. Normally, the whole histogram is just one colour (here they are colored to be understood easily), so one gets almost no information from it except the size. But if this is combined with side scatter histogram, shown in Figure 2.37(B), which also does not tell us much except the cellular complexity, then a two dimensional scatter histogram/plot, shown in Figure 2.37(C), can be obtained. This is called a *two dimensional dot plot* where every dot represents an event/cell on the plot. As shown in the figure/plot, it can be clearly seen that each cell types discriminate itself from each other.

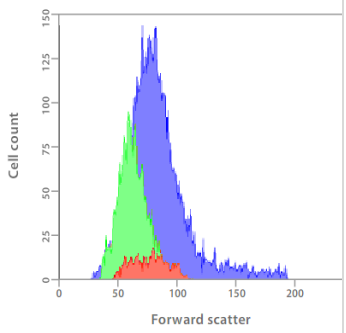


Figure 2.37(A): *1D Forward scatter histogram for three different cell types[35].*

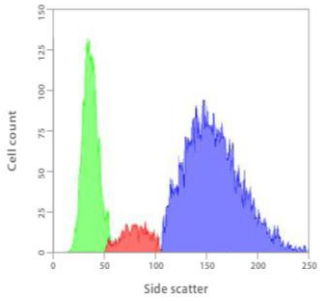


Figure 2.37(B): *1D Side scatter histogram for three different cell types[35].*

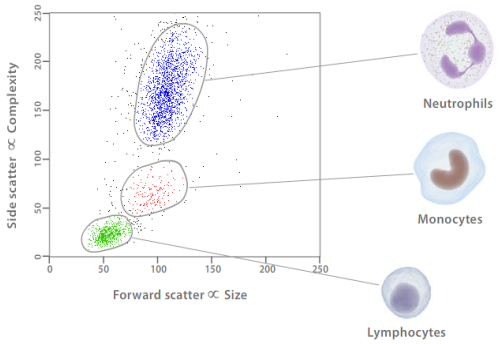


Figure 2.37(C): *2D Forward scatter histogram for three different cell types marked with three different colours[35].*

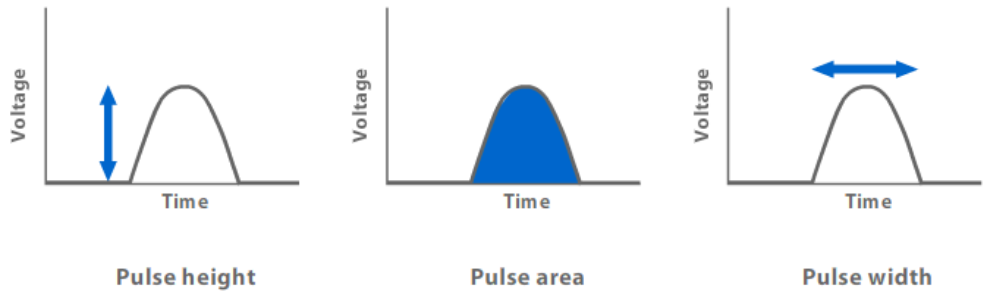


Figure 2.38: *Illustration of a voltage pulse with its pulse height, pulse area, and pulse width[35].*

The voltage pulses are converted into numerical values and this is done in three ways for each cell/event: measuring pulse height, pulse area, and pulse width, shown in Figure 2.38. To measure the amount of DNA, usually *FL-2 (PI) width* verses *cell count (event)* is used to avoid two attached nuclei from single nuclei.

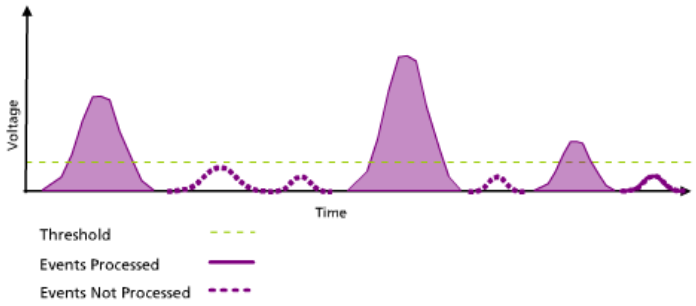


Figure 2.39: *Applying a threshold to voltage pulses[28].*

Figure 2.39 illustrates a threshold voltage, shown in dashed lines, which tell the computer which cells should be processed or not. Every generated signal under the threshold is not processed and the rest is processed.

2.5.4.2 Spectral Compensation

Since there are different PMTs for each colour (each fluorophore), it is desired that PMT detector collects light only from that specific fluorophore. But in reality, a PMT detector collects photons from another fluorophore as well at the same time in the same colour. This means the data collected by that detector is wrong. It must be corrected / compensated.

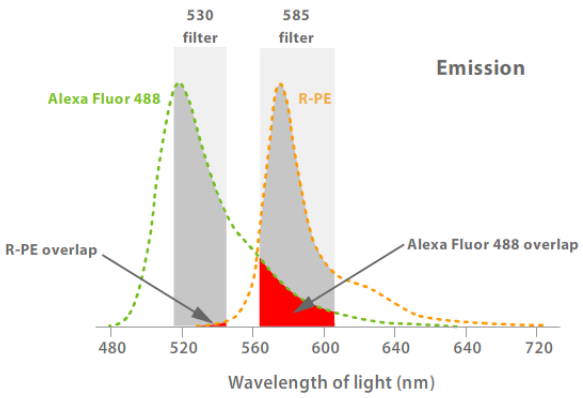


Figure 2.40: *Emission spectra of Alexa Fluor 488 and of R-Phycoerythrin (R-PE) fluolophores[35].*

On the left (Figure 2.40) emission spectra of Alexa Fluor 488 and of R-PE are demonstrated. In a perfect world, these emission spectra would be very intense narrow peaks separated from all other emission peaks. In reality, organic dyes and fluorescence proteins have broad emission peaks and their both excitation and emission spectra can overlap each other. To avoid this overlapping, first *emission band-pass filters*, shown in rectangles in the figure, are used. This basically tells the optical system to direct only the photons inside these rectangles to the correspondents PMTs.

But this is still not enough to correct data because there is still overlapping marked with red colour inside both rectangles. If one filters more, more fluorescence will be lost. The effect of this overlapping on the voltage pulses will be an increase in the pulse height, pulse width, and pulse area. For each event by subtracting a percentage of fluorescence in the Alexa Fluor 488 channel from fluorescence in the R-PE channel will give us the compensation/correction from the contribution of Alexa Fluor 488 to the R-PE and vice versa for the compensation of R-PE to the Alexa Fluor 488. To determine the amount of compensation for each fluorophore,

control sample stained with each single colours (Alexa Flour 488 or R-PE) are analysed in parallel with the experimental samples stained with multiple fluolophores. Compensation can be performed before the sample is run or after data collection. The compensation performed during this master project is presented in *Appendix B.*

2.5.4.3 Gating

In flow cytometry *gating* means a selection of cells (according to their fluorescence and /or scatter characteristics) that will be carried forward for further analysis. It is generally acknowledged to be one of the most powerful, but also one of the most problematic, aspects of flow cytometry. It is problematic because flow cytometrists like to think of their technique as objective and do not like to admit that much of flow cytometric analysis rests on the foundation of a few subjective initial decisions. The ideal strategy for gating should therefore be: *First*, gating needs to become as objective as possible, and *second*, flow cytometrists need to recognize explicitly those aspects of gating that continue to require subjective decisions[32].

Gating is powerful because it can show sub-populations inside a sub-population for further evaluation. If you remember, in histograms section, it was assumed that there were three different cell types with different sizes and granularities without fluorophore molecules to plot a 2D forward – side scatter dot plot as in Figure 2.37 (C).

Now if one assumes that two different fluorophore –labelled antibodies which bind themselves to CC4 (R-PE fluorophore) and CCD8 (R-PE Alexa Fluor 700) antigens for lymphocytes are added to the same sample then it is possible to select the cell of the interest by applying a gate. As shown in Figure 2.41(A), one can draw a rectangular gate highlighted with green colour around this sub-population (lymphocytes). As a result of this gate, it excludes other cell types (monocytes and neutrophils) and includes only lymphocytes for further analysis. On the right side of the figure, it is seen what kind of lymphocyte combinations are present after staining with two fluorophores; cells with CC4, cells with CC8, cells with both CC4 and CC8, and cells with no fluorophores.

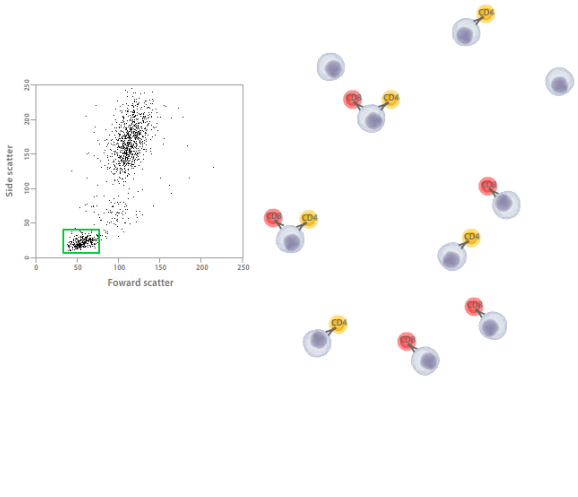


Figure 2.41 (A): *Gating for lymphocytes [35].*

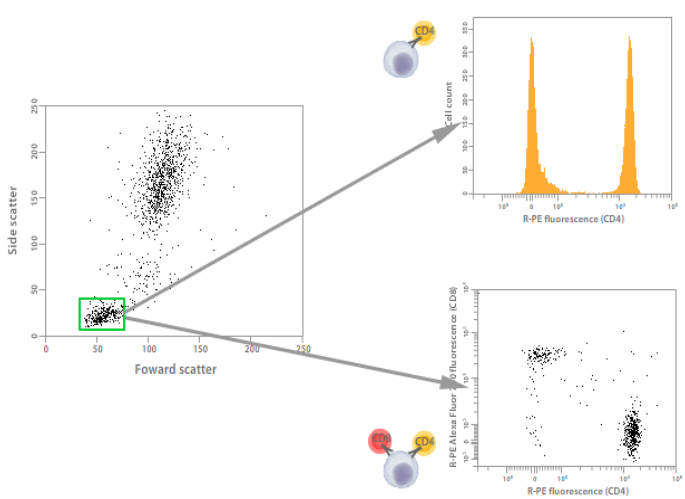


Figure 2.41 (B): *Gating for lymphocytes – fluorescence for single antigen or two antigens on a 2D dot plot[35].*

These sub combinations can be plotted with additional plots, such as cells with CC4 (single antigen) or both CC4 and CC8 (two antigens), shown in Figure 2.41 (B).

2.5.4.4 Quality Control

There are many factors which affect the quality of a histogram. One of them is the *sample preparation* in which the aim is to obtain single particles, cells or nuclei with minimum degradation of their DNA and minimum clumping. Sample preparation can be controlled by using a microscope. If there is a lot of debris or clumps, the sample preparation method should be re-evaluated. Cell clumping can take place during fixation especially in 70% ethanol and it can be reduced either by passing the sample through a 26 gauge needle or by filtering the cells through 50-80 µm nylon mesh[34].

Another factor is the *instrument alignment* which should be checked before the experiment using fluorescent beads prepared for this purpose. For the DNA analysis using suitable beads, a cv of 1.5% or better should be obtained. If the value is bigger than this, there may be dirt in the flow chamber. **Coefficient of variation (CV)** is a common measure of the spread of a distribution and it is given by following equation:

$$CV = \left(\frac{SD}{MEAN}\right)100 \tag{2.12}$$

where SD is the standard deviation and the MEAN is the arithmetic mean or average value for the parameter measured for these particles. For a Gaussian distribution this is the channel with the highest count[27]. CV is important because it is independent of channel number, that is, dimensionless and it allows samples from different instruments on different settings to be compared directly.

Staining procedure is another factor which affects the quality of a histogram. When the cells are stained with a DNA-binding dye like PI, enough time must be given to the RNase to remove all double-stranded RNA and for the dye to equilibrate with the same. If the fixed cells/samples are left overnight in the cold, the quality of the DNA histogram will be often improved. If the concentration of the cells is very high, then the dye could be insufficient to maintain stoichiometry.

The quality of a DNA histogram is obtained by measuring the width of the G_O/G_1 peak. Small cvs mean better resolution of small differences and more reliable cell-cycle distribution components. Normal lymphoid cells from blood or bone marrow can give cvs close to 1% while tumour samples or cultured cells usually have bigger cvs and a cv of 2.5 % is considered a typical best cv for these cells[34]. A cv bigger than 8% is considered as “not good”[32]. If the cell-cycle distribution would be analysed, then minimum 10 000 events are needed[34]. Finally, the flow cytometry measurements should be run at slow mode since fast mode can lead to higher cvs.

2.5.5 DNA Content Analysis

2.5.5.1 Cell Cycle

The cell-cycle distribution can be monitored according to the content of DNA in the cells using flow cytometry with a DNA-binding dye. The signals generated in the detector while the cycling cells with different DNA content pass through a laser beam are presented in Figure 2.44. The cells in G_0 and G_1 are considered to have the same amount of DNA and it is labelled as 2C (*diploid*) shown in Figure 2.45. The cells in G_2 and M have double DNA content than those in G_1 and G_0 and labelled as 4C (*tetraploid*). The ones in S phase have DNA content between 2C and 4C. An ideal theoretical DNA histogram for cycling cells would be as in Figure 2.45(A) but in reality Figure 2.45(B) pops up due to having no perfect flow cytometer and no perfectly uniform binding of the DNA-specific dye[39]. If we had these conditions in real life, for example, the G_1 peak would be represented by only one channel number. But in reality it is represented by many channel numbers which show a Gaussian distribution as in G_1 and G_2. S phase shows no Gaussian distribution therefore to estimate the proportion of S phase cells, one of the four different models based on sets of assumptions is used. They are *peak reflect method*, *rectangular approximation*, *S-FIT model*, and *the sum-of-broadened-rectangles (SOBR)[32]*.

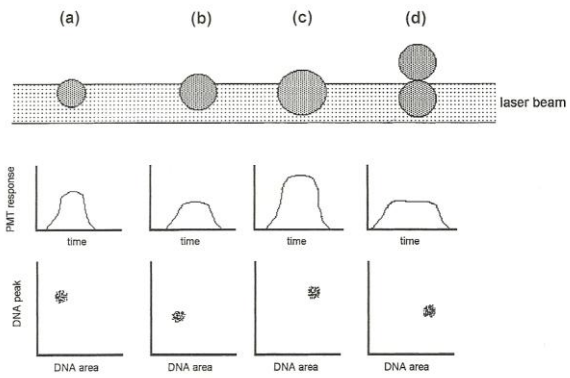


Figure 2.44: *The shapes of the signals produced as different fluorescent nuclei cross a laser beam.(a) A compact nucleus in G1; (b) a normal nucleus in G1; (c) a nucleus in G2; (d) two G1 nuclei clumped together[34].*

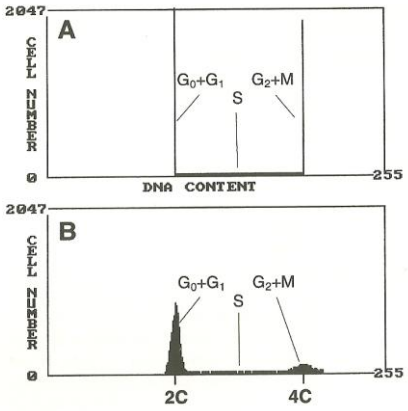


Figure 2.45: *Ideal (A) and real (B) DNA content distributions with the same ratios of cells [31].*

The problem in S phase rises due to overlapping between G_1 and S, and between S and G_2.

2.5.5.2 Ploidy

Different organisms have different amount of DNA in their cells. Human cells contain almost 6 pg of DNA per nucleus while it is 2,5 pg for chicken cells. For corn cells it is about 15 pg while for *Escherichia coli* it is just between 0.01 and 0.02 pg[32]. All healthy cells in all organisms contain the same amount of DNA all the times except three major exceptions: during meiosis (cells contain 1C=*haploid* nuclei), during S phase (from 2C to 4C) and during apoptosis (losing pieces of fragmented DNA).

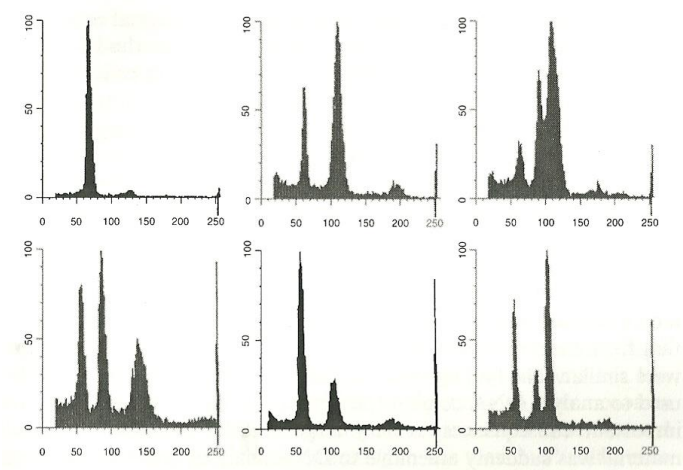


Figure 2.51: *PI fluorescence histograms from nuclei aspirated from normal tissue (upper left) and malignant breast tumours [32].*

The cells which are said to be normal are called *euploid* or normal diploid while abnormal cells are termed *aneuploid*.

If there are some types of abnormalities associated with genetic changes, these changes may sometimes be reflected in changes in total DNA content of the malignant cell. In Figure 2.51, a normal tissue is shown at upper left and the rest histograms belong to malignant breast tumours. An abnormal peak or peaks may have more (*hyperdiploid*) or less DNA (*hypodiploid*) than normal cells. Sometimes it is possible to see that a single peak with a wide cv or distorted profile may cover a near-diploid malignant cell line or an extra small peak at the 4C position may result from clumping of nuclei, cycling cells, or a true tetraploid tumour.

2.5.6 Principles of the Vindeløv Method

In this master project, two different types of DNA content analysis experiments were performed. The first one was the Vindeløv method which directly analyses the DNA content from the nuclei. To obtain the nuclei from the whole cells, a detergent –trypsin technique was used. The advantage of the Vindeløv method is to deal just with the nuclear DNA because cells have not only nuclear DNA but also mitochondrial DNA[2]. The other type of the experiment was to analyze both the DNA content and the amount of protein which is why the cells were treated as whole since the proteins are inside the cytoplasm.

Vindeløv method was developed and established by Lars L. Vindeløv and his other two colleagues, J. Christensen and Nils I. Nissen in 1982 in Copenhagen. Their four articles[40-43] were accepted on September 28 in 1982 by CYTOMETRY journal. The method developed and established in this project based on their article with page number 323-327[40]. Before this article, they had managed to develop a simple procedure for long-term storage of cells for flow cytometric DNA analysis, up to one year by freezing them at -80 ^{0}C[41]. This long – term storage of samples were integrated into their method, which was developed in this project: *A Detergent- Trypsin Method for the Preparation of Nuclei for Flow Cytometric DNA Analysis*.

Since determination of nuclear DNA content by flow cytometry requires comparison with a reference standard, they developed a method to solve this problem by help of using two internal standards, Chicken red blood cells (CRBC) – have a DNA content of 35 % of the human diploid value, and Rainbow trout red blood cells –have a DNA content of 80 % of human diploid cells. A mixture of these two cell types was prepared and store in small aliquots at -80 ^{0}C. Later this mixture was added to the sample before staining[42].

Using two internal standards were better than one because DNA content (DNA ratio) is very sensitive to changes in the zero level adjustment of the flow cytometer. If two internal standards are used the DNA ratio becomes independent of the zero level[42].

In their next article[43], they identified the limiting factors of detection of nuclear DNA abnormalities by flow cytometric DNA analysis. They analysed 240 samples of leucocytes from six men and six women. The DNA content of female cells was 1.5 % higher than that of male cells, which is known as sex related DNA difference. In addition to the sex related differences in DNA content, individual differences of up to 1 % were also observed. Another limiting factor was tissue related differences in fluorescence which adds uncertainty to the interpretation of the results. Moreover they wrote that a lower coefficient of variation would increase the resolution, but tissue related differences in fluorescence could then become the limiting factor[43].

3 EXPERIMENTAL PART

3.1 Materials and Methods

In this part, the materials which were used and the methods which were performed and developed will be presented.

3.1.1 Materials

3.1.1.1 The Cell Lines

Normal cells can divide themselves for a certain number of times and eventually get older and die. In tissue studies in vitro therefore it is necessary to work with the cells which can divide themselves for an infinite number of times as long as the favourable conditions are provided.

In the experiments, two main cell lines which are denoted T-47D and T98G were used. In addition to this, cells exposed to different pre-treatments were used: T98G had three sub lines: *T98G-LDRres* (Low Dose Rate resistant), T98G-P (Primed) while T-47 had: T-47D-P, T-47D-P4, F10, and F44. More details about these cells can be found in Table 3.1.

The **T-47D cell line** was established from the pleural effusion of a patient with breast carcinoma in1974. The cells have epithelial morphology and form monolayers in culture[44]. The cells have normal Rb-fuction [45] and contain a mutation in gene which codes for p53 therefore they are unable to perform normal p53-function[46]. T-47D cells show HRS[23] and under normal favourable growth conditions they can double themselves exponentially with a doubling time of 37.2 ± 2.0[47].

The **T98G cell line** was originally derived from human glioblastoma tumour tissue of a 61-year-old Caucasian male and the cells were named as *T98*. Some samples of this cell type were frozen after 25 population doublings. Other samples of T98 were cultivated 300 population doublings. These two populations of cells showed different characteristic therefore the cells after 300 population doublings were termed as T98G[48]. Normal cells can undergo only a limited number of population doublings. They cannot proliferate with anchorage and they become arrested in G_1 under stationary phase conditions. T98 differ from normal cells in all these properties. But T98G cells, the derivative of T98, are like normal cells in that they become arrested in G_1 under stationary conditions, they also exhibit the transformed characteristics of anchorage independence and immortality[48]. T98G has about double as many chromosomes as T98[48]. The cancer in the central nervous system because of this cell type is the most common in adults and a patient with this cancer lives approximately 1 year because the cells of this type are resistant to chemotherapy[49]. T98G cells can be bought on internet, for example, from Sigma-Aldrich. They store the cells at -190 ^{0}C[50].

The cell types used in the experiments and their explanations are given in Table 3.1.

Table3. 1: The cell types used in the experiments and their explanations.

Cell type	**Explanation**
T98G	Mostly used as control
T98G LDRres	*T98G LDRres (T98G Low Dose Rate resistant):* The cells which grown in [^{3}H]-medium with a specific activity of 1.67 μCi/ml for 10 months
T98G-P	*T98G – P (T98G -Primed):* The cells which were irradiated with 0.3 Gy/h during 1 hour then growing for 9 months without radiation.
T-47D	Mostly used as control
T-47D LDRres	T *T-47D LDRres (T-47D Low Dose Rate resistant cells):* The cells which growth in [^{3}H]-medium with a specific activity of 1.67 μCi/ml for 10 months
T-47D –P	*T-47D – P (T-47D-Primed):* The cells which were irradiated with 0.3 Gy/h during 1 hour then grown for 4 years without radiation.
T-47D –P4	T-47D-Prime: The cells which were irradiated with 0.3 Gy/h during 1 hour then 3 months growing without radiation.
F10	*F10 (T-47D):* The cells which were grown in [^{3}H]-medium (0.46 ml [^{3}H] in 150 ml) with a specific activity of 1.6 μCi/ml for 5 months and 13 days. Then they were grown 17 days without radiation before they were frozen on 17.02.2003. F10 is the code/name used for these cells in the freezer journal at Biophysics Cell Laboratory.
F44	*F44 (T-47D):* The cells which were grown in 4% hypoxia and in [^{3}H]-medium with a specific activity of 1.67 μCi/ml for 35 days. Hereafter they were grown for 45 days without hypoxia and irradiation until they were frozen on 16.09.2009. F44 is the code/name used for these cells in the freezer journal at Biophysics Cell Laboratory.

3.1.1.2 The Vindeløv Method

This method uses five solutions which are: 1- *Stock Solution*, 2- *Solution A (s)*, 3- *Solution B*, 4-*Solution C*, and *5- Citrate Buffer*. To clean the medium and trypsin *6- Phosphate buffered saline (PBS) solution or 7- 0.9 % NaCl* salt were used in the experiments. The solutions for Vindelöv method are presented as following:

1- Stock Solution	
• Trisodium citrate 2 H_2O • Tergitol soap • Sperminetetrahydrochloride • Tris (hydroxymethyl)-aminomethane • Total volume (milli-Q water) • PH adjusted to	2000 mg (3.4 mM) 2 ml (0.1 % v/v) 1044 mg (1.5 mM) 121 mg (0.5 mM) 2000 ml 7.6
2- Solution A1	
• Stock Solution • Trypin (Sigma, T0134) • PH adjusted to	500 ml 15 mg 7.6
3- Solution A6	
• Stock Solution • Trypin (Sigma, T0134) • Triton-X 114 soap • PH adjusted to	100 ml 3 mg 50 µl (0.5 % v/v) 7.6
4- Solution A8	
• Stock Solution • Trypin (Sigma, T0134) • Triton-X 114 soap • PH adjusted to	100 ml 3 mg 100 µl (0.4 % v/v) 7.6
5- Citrate Buffer	
• Sucrose • Trisodium citrate 2 H_2O • Dimethylsulfoxide zur syntese (DMSO) • Total volume (milli-Q water-distilled water)	85.50 g (259 mM) 11.76 g (40 mM) 50 ml 800 ml
6- PBS	
• KCI • KH_2PO_4 • Na_2HPO_4 x 2 H_20 • Total volume (milli-Q water) • PH adjusted to	0.402 g 0.408 g 2.84 G 2000 ml 7.3
7- NaCl	
• NaCl • Total volume (milli-Q water	0.9 g 100 ml

3.1.1.3 The two-parametric DNA – Protein Method

The solutions for the DNA-Protein method are presented as following:

PBS (1X)	100 ml PBS (10X) in 900 Milli-Q water
Rectified Spirit	Rectified Spirit (Ethanol)
RNase Solution	RNase (60 µg/ml) in PBS (1X)
FITC Solution	FITC (0.1 µg/ml) in PBS (1X)
PI Solution	PI (34 µg/ml) in PBS (1X)

3.1.2 Methods

3.1.2.1 Cell Cultivation

The cells utilized in these experiments were grown in the Biophysics Cell Laboratory at the Department of Physics, University of Oslo (and some of them irradiated with ^{60}Co γ–radiation at the Norwegian Radium Hospital.). To maintain sterility and safety of the cells and the human, a sterile LAF (Laminar Flow) Bench was used. The surface of the bench was disinfected with 70 % ethanol before and after use. Equipment and chemicals which would come in contact with the cells were sterilized before use. The cells were grown in 25 cm^2 sterile plastic culture flasks (Nunclon, Denmark) as monolayer cultures in RPMI (Roswell Park Memorial Institute) 1640 growth medium supplemented with 10 % fetal calf serum (Gibco), 2 mM L- glutamine (Sigma), 0.2 % insulin (Sigma), 1 % penicillin (Gibco), 1 % streptomycin (Gibco) in air containing 5 % CO_2. The medium contains all the nutrients, vitamins and salts which the cells need in order to survive. The fetal calf serum and insulin add the growth factors which are necessary for proliferation and the penicillin and streptomycin are antibiotics to prevent the growth of bacteria because microorganism like bacteria, virus, fungi and mycoplasma can divide themselves almost 50 times faster than human cells[51]. The doubling time of T-47D is 37.2 ± 2.0[46] and it is 24.42 ± 1.7 for non-irradiated T98G cells[16]. RPMI 1640 contains pH indicator phenol red which changes colour from yellow to red when pH exceeds about 7.4. Optimal pH values for cell growth is 7.2 – 7.5[51].

Growing cells need regularly fresh supplies of nutrients and growth factors in order to maintain a stable growth. At the same time their toxic waste must be taken away from the cellular environments. It is also important to control the cell density since both too high and too low cell densities can inhibit the cell growth. The cells were kept in exponential growth by reculturing of stock cultures twice a week, Monday and Friday and change of the medium every Wednesday. This was done by Joe A. Sandvik, Ida Aspmodal, Stine Christoffersen and Celal Ceyhan. The lid of the medium glass flask and the area of the glass flask touched to the lid were burned slightly to inhibit infections each time before and after use.

At *recultivation*, the old medium was first removed, later the cells which were attached to the bottom of the plastic flasks were rinsed by 2 ml trypsin. This 2 ml was removed and a new 3 ml trypsin (for 25 cm^2 flasks) was added. Then the flask with closed lid was put into an incubator which had 5 % CO_2 , 95 % humidity and 37 ^{0}C temperature. After some minutes,

the cells were taken out from the incubator and checked by a microscope (Nikon TMS, Japan) to be sure if they were loosened by the trypsin. Trypsin is a proteolytic enzyme which breaks the protein bonds between the cells and the bottom surface of the flask that they are attached to, and between neighbouring cells. The effect of trypsin was enhanced by adding EDTA which causes the bonds among the cells to be loosened by binding itself to the Ca^{2+} which the cells are dependent on to be able to maintain the intracellular bonds. When the cells are loosened, they can be transferred to new flask with medium by help of a pipette. The flask was kept in the incubator with the lid of the flask half closed.

3.1.2.2 Cell Counting

In some experiments, two different cell types or nuclei were mixed in order to compare the contents of the DNA and protein. Therefore it was necessary to count the cells or nuclei for a good comparison.

3.1.2.2.1 By Bürker Chamber

The cells or nuclei were taken out from a solution and inserted into one of these chambers shown in Figure 3.1(A). Each chamber has 3x3 big squares where each big square contains 9 small squares shown in Figure 3.1(B). The cells or nuclei in the 9 big squares were counted and the biggest and the smallest numbers were ignored. The same thing was repeated for another chamber and the average values of the two chambers were calculated. The volume of each big square is 10^{-4} ml. By multiplying each big square with 10^4, the total number of the cells/nuclei per each big square was obtained.

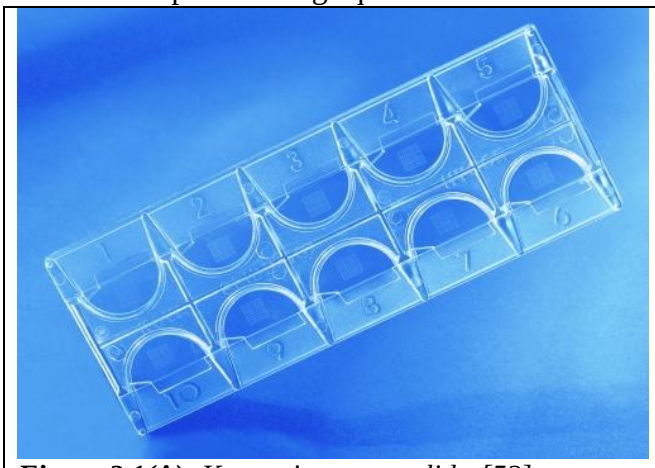


Figure 3.1(A): *Kova microscope slides[52].*

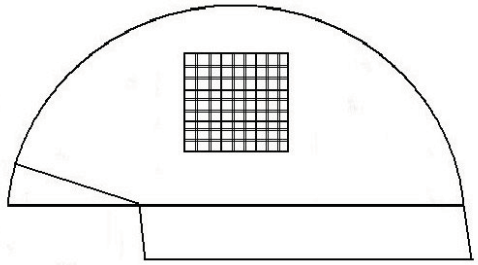


Figure 3.1(B): *Illustration of one Kova chamber[16].*

3.1.2.2.2 By Flow Cytometry

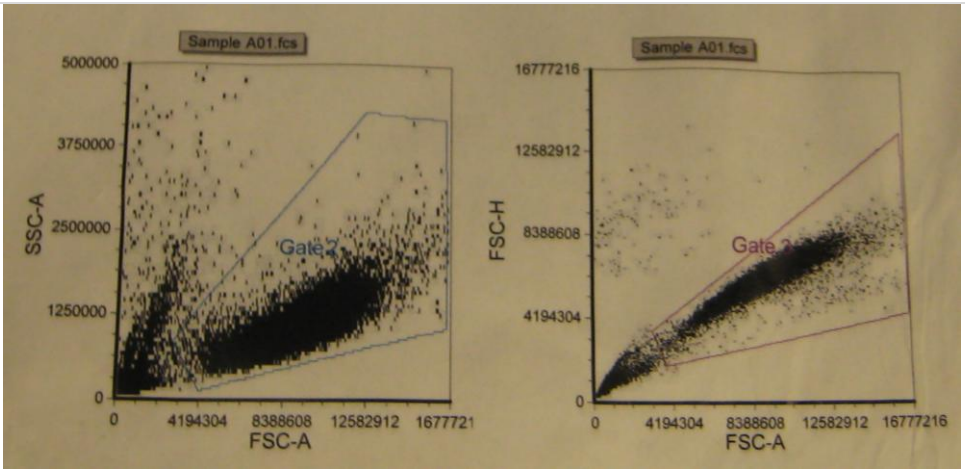


Figure 3.2: *Cell counting by flow cytometer*.

The sample tubes had 0.5 ml from each sample cell type. Filtering and vortexing the sample tubes were performed before running the samples by the flow cytometer at fast mode to avoid extra big particles. The cytometer was programmed to count the cells for 200 µl so the count was multiplied with 5 to find the cell/nuclei number per ml. A gate as seen in Figure 3.2, was applied to FSC-A - SSC-A and FSC-A - SSC-H to order the computer to count only the cells. For each cell type, the whole process was repeated twice and the average value was used.

3.1.2.3 The Vindeløv Method

The Vindeløv Method to prepare nuclei for flow cytometric DNA analysis is summarized in Figure 3.3(A). During this master project, the flow sheet was modified to be able to establish the method in the Biophysics Cell Laboratory.

There were basically three main changes:

- *NP -40* soap was replaced by Tergitol, SDS and Triton X-114 soaps and in the end Triton X-114 was chosen for further analysis.
- Solution A time was changed from 10 minutes up to 15 (later 22 in last two experiments) minutes for T98G cell line and 22 minutes for T-47D cell line.
- PBS was replaced by 0.9 % NaCI salt.

The final and modified method/protocol is presented below used in this project is shown in Figure 3.3(B).

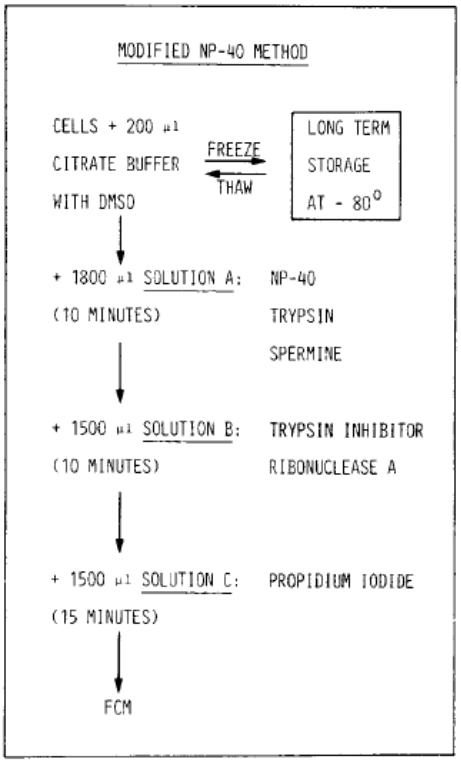


Figure 3.3(A): *The original flow sheet and salient features of the modified preparation of Vindeløv method[40].*

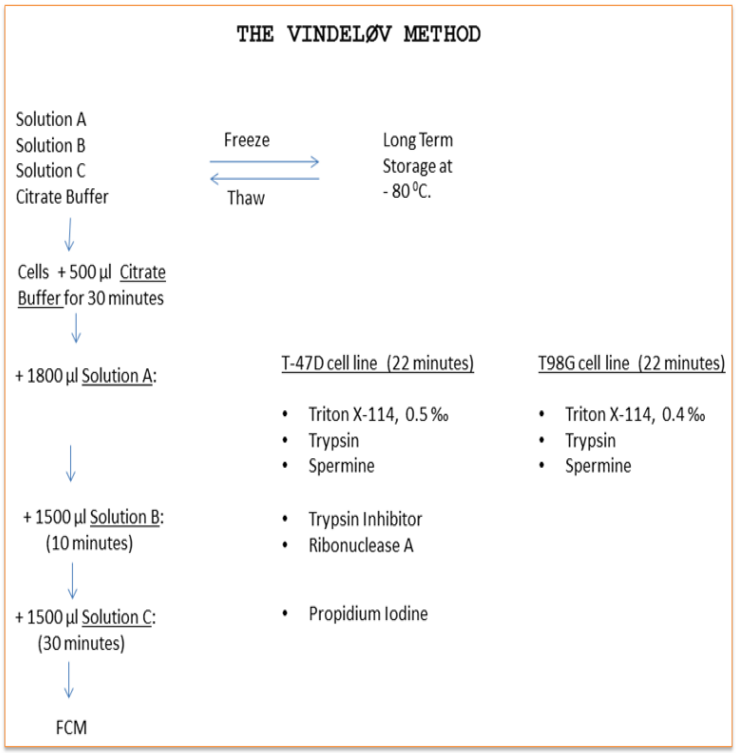


Figure 3.3(B): *The modified Vindeløv method used in this project.*

3.1.2.4 The two-parametric DNA – Protein Method

This method is mainly based on the study of Øystein W. Rønning and Tore Lindmo[53]. Briefly, the cells were trypsinized and fixed in 50 % ethanol (Rectified Spirit) in phosphate-buffered saline (PBS (1X)) and left overnight in a freezer. Next day, the fixed cells were centrifuged and resuspended in 3 ml PBS containing 0.1 µg/ml FITC (fluorescein isothiocyanate) in PBS (1X) and left overnight in the dark at room temperature. The following day, the FITC (also PBS(1x)) was removed from the cells without cleaning with PBS (1X).

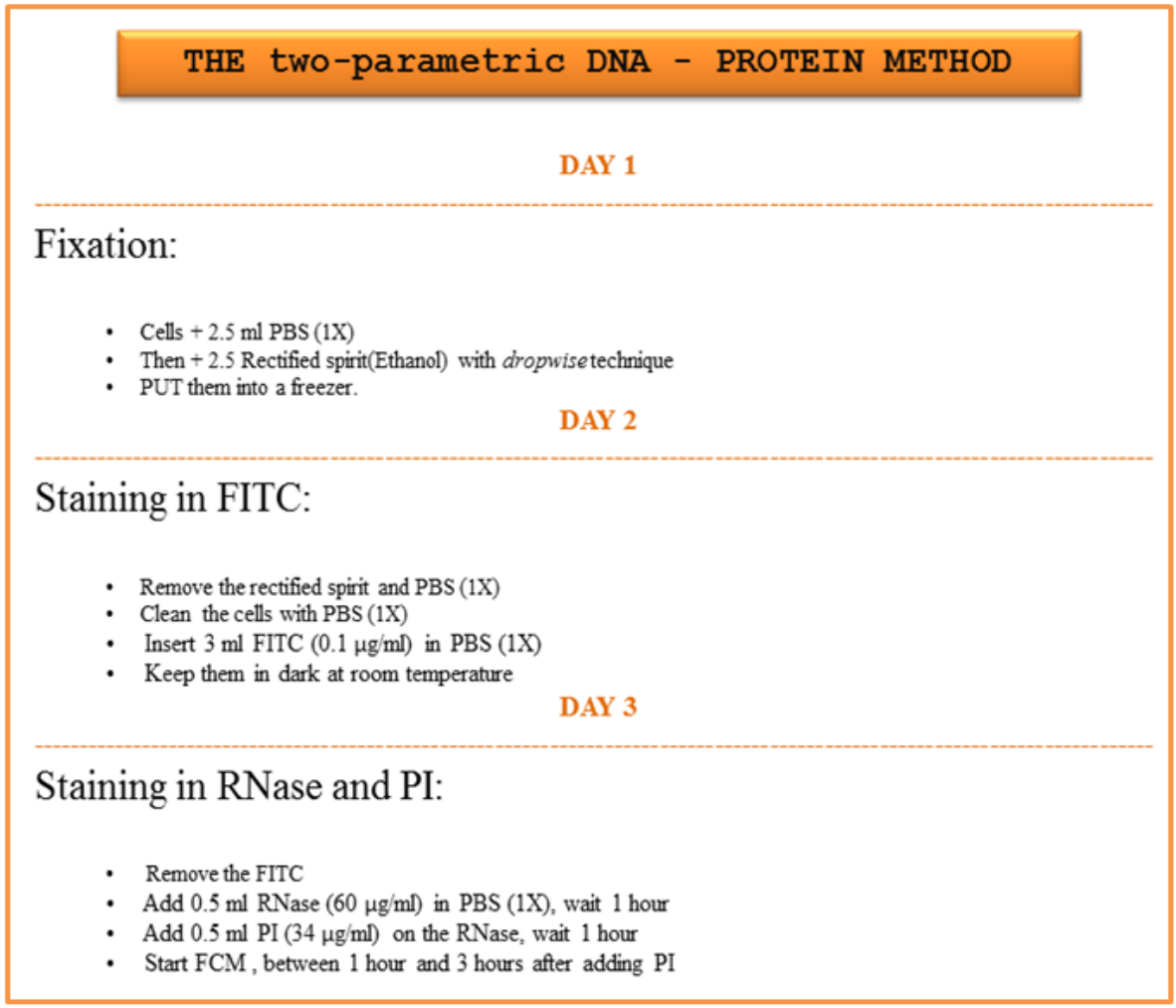


Figure 3.4: *The DNA-Protein method.*

Thereafter, the cells in each tube were resuspended in 0.5 ml RNase (60 µg/ml) in PBS (1X) for 1 hour. After 1 hour, 0.5 ml PI (34 µg/ml) in PBS (1X) was added to the each tube which already contained 0.5 ml RNase (60 µg/ml) in PBS (1X). The cells stained with PI for 1-3 hours were ready for flow cytometric measurements. Since the cells contained both still some FITC and PI, due to energy transfer from FITC to PI a certain fraction (approximately 15 % [53]) of green (FITC) signal had to be subtracted from the red (PI) signal. This was according to [53]. But in the experiments in this project, the spectral compensation rate was between 6.5 % and 8.5% using FCS Express software program.

3.2 Results

During this project, there were two main goals to achieve. The first one was to obtain nuclei from whole cells using the method which was developed by Vindeløv. Thereafter it was to apply the method to different type of cells to analyse them. The second one was to establish a method to analyse the DNA content versus the protein content. To reach the first goal, 19 experiments were done and for the second goal it was 8 experiments. The second one was based on the study of Rønning and Lindmo[53].

3.2.1 The Vindeløv Method

This part was divided into two parts. *1- Develop and establish the method*, *2- Apply the method to cells with different characteristics and backgrounds*. To show 1-, it is necessary to write down step by step what was done/changed in the experiments.

The first problem with the Vindeløv method was to find the soap, *NP-40*, which he used. Because it was not produced any more the soap had to be replaced by another soap. The Stock solution1 and solutions A, B, C and Citrate Buffer were prepared by Nina Edin and Celal Ceyhan and a soap called Tergitol was used to prepare these solutions. After preparation, they were stored in a -80 ^{0}C freezer. These solutions (B, C, Citrate Buffer) were used during the whole project except solution A1 and stock solution1 which were not utilized in some experiments. Look at Appendix A1 to learn more details about the experiments where the solutions were given numbers/names.

The first experiment was done on 8.2.2011 using T-47D, T-47D-P, T98G, and T98G-P cells. No flow cytometry measurements (FCMs) were performed only the photos of the cells after treatment with solution A (also B and C)were taken which are shown in Figure 3.5(A-D).

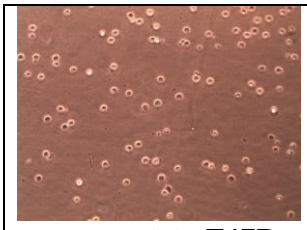


Figure 3.5(A): *T47D*

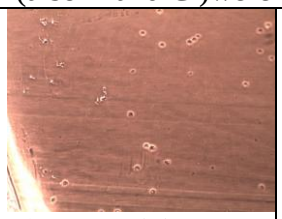


Figure 3.5(B): *T47D-P*

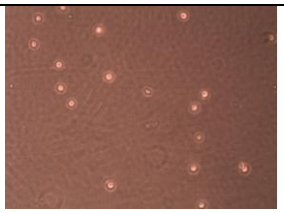


Figure 3.5(C): *T98G*

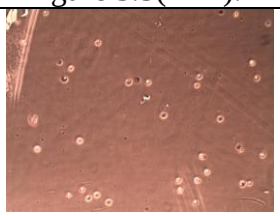


Figure 3.5(D): *T98G-P*

As the figures demonstrate, what was done in the first experiment was not enough because there were a lot of whole cells, clumps and just a few nuclei. PBS was used to clean the cells from the medium and trypsin-EDTA, and the Tergitol concentration was 2 ml per 2000 ml as described in the method for NP-40 soap.

In the second experiment, Tergitol concentration was increased from 0.1 % to 40 % with preparing solution A2 from the Stock solution1 since a lot of it had been stored before. T98G and T-47D cells were utilized this time. The result was not good enough and since high soap concentration could damage the cytometer, it was decided that the soap must be changed.

In the third experiment, 1 litre of Stock Solution2 without soap was prepared. Solution A3 without soap using Stock solution2 was prepared. Later this solution A3 was mixed with Triton-x soap with 0.001 % concentration to prepare solution A4. Thereafter the solution A3 and the solution A4 were mixed at rates of 1 to 4 and 0.5 to 4.5, respectively to produce new solution As. The results were not good enough.

In the fourth experiment, 0.9 % NaCl in MilliQ water started to be used instead of PBS. A new soap, Sodium dodecyl sulphate (SDS), was utilized mixing with either Tergitol soap from stock solution1 or Triton-x from stock solution2. In addition, both Triton-x and Tergitol were used alone using either T-47 or T98G cells. First time FCM measurements were performed and the results were not satisfactory.

In the fifth experiment, 0.1% Tergitol versus 0.001% SDS in solution A5 was used together with either Tergitol or SDS in another solution A5. Solution A5 time varied from 10 up to 20 minutes. Citrate Buffer time was decreased from 30 to 20 minutes. *In the sixth experiment*, the same solutions as in experiment 5 were used with changing solution A5 time; either 20 or 35 minutes. Citrate Buffer time was increased to 30 from 20 minutes. *In experiment 7*, T-47D and T98G cell lines were left overnight in 500 µl CB in – 80 ^{0}C freezer. Later the cells with the same solution A5 were stained inside solution A5 during 20 minutes at 37 ^{0}C. *In experiment 8,* everything was the same as experiment 7 but the cells were also stained at room temperature (20 ^{0}C) in addition to staining at 37 ^{0}C in solution A5.

In experiment 9, T98G-p cells were heated up to 37 ^{0}C and stained in the same solution A5 for 22 minutes. The same thing was performed at room temperature as well for 42 minutes in solution A5. The results both from the photos and the FCM showed that the samples heated up to 37 ^{0}C were better than the samples stained at room temperature(20 ^{0}C) as shown in figures 3.6 (A-D). Therefore *in experiment 10*, all the samples were stained in solution A5 at 37 ^{0}C during either 22 minutes or 25 minutes for both T-47D and T98G-P cells.

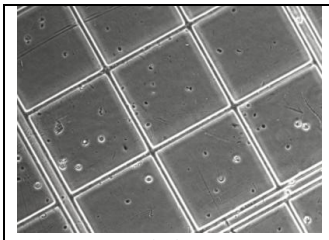


Figure 3.6(A): *T98G-P after22 minutes in solution A5 time with 37 ^{0}C.*

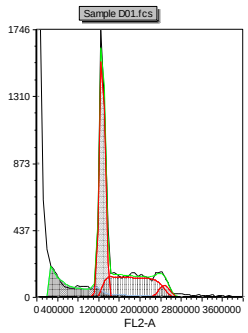


Figure 3.6(B): *T98G-P 22 after minutes in solution A5 time with 37 ^{0}C. G1_CV is 4.05 ±0.01.*

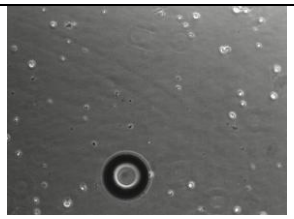


Figure 3.6(C): *T98G-P after 22 minutes in solution A5 time with 20 ^{0}C.*

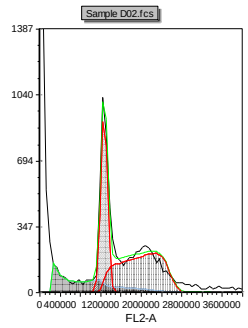


Figure 3.6(D): *T98G-P after 22 minutes in solution A5 time with 20 ^{0}C. G1_CV is 5.05±0.01.*

The results for T-47D were not good enough to be considered. The T98G-P cells stained in solution A5 for 22 minutes had an average G1_CV of 3.82 for different staining times in solution C while the cells stained for 25 minutes had G1_CV of 4.69. When the same staining time in solution C (30 minutes) was compared, the cells stained for 25 minutes had G1_CV of 3.69 while it was 4.0 for the cells stained in solution A5 for 22 minutes. Since the photos showed that there were more whole cells than nuclei, it was decided that a new soap must be used.

In experiment 11, solution A6 and solution A7 were prepared using stock solution1 and Triton-X 114 with different concentrations (0.25 ‰, 0.5 ‰, and 1 ‰). This time only T-47D cells were utilized using 1‰ and 0.5 ‰ Triton-X 114 soap with 22 minutes staining in solution A6 or solution A7 both for 20 ^{0}C and 37 ^{0}C. No FCMs were performed but photos were taken as shown in figures 3-7(A-B). Finally the method for T-47D cells was almost established. The pictures for 1 ‰ Triton-X 114 for 20 and 37 are not shown because of bad results.

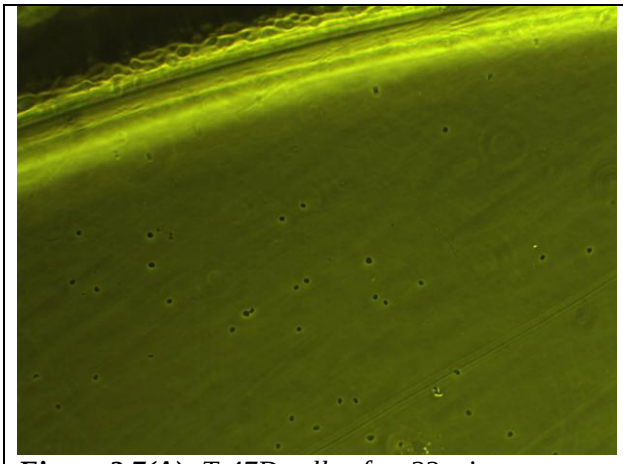


Figure 3.7(A): *T-47D cells after 22 minutes staining in solution A of 0.5 ‰ Triton-X 114 at 20 ^{0}C.*

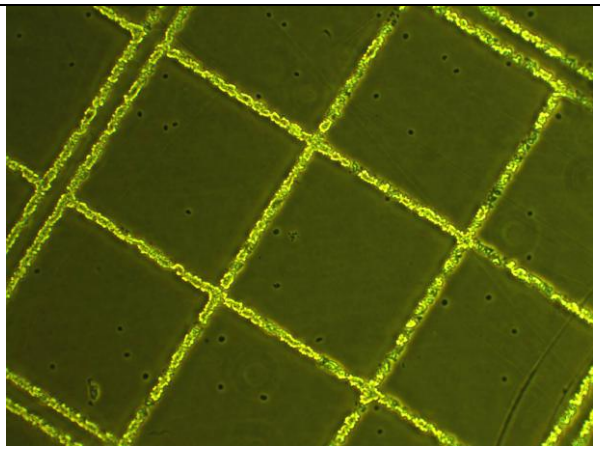


Figure 3.7(B): *T-47D cells after 22 minutes staining in solution A of 0.5 ‰ Triton-X 114 at 37 ^{0}C.*

In experiment 12, the same thing as in experiment 11 was repeated for T47-D cells using 0.5 ‰ Triton –X 114 in solution A6 with 22 minutes staining time both for 20 ^{0}C and 37 ^{0}C to be sure if the same results could be obtained again. For T98G cells the same concentration (0.5 ‰) with 22 minutes staining time in solution A6 both for 20 ^{0}C and 37 ^{0}C was used while solution A1 made of stock solution1 was performed for only 37 ^{0}C. The G1_CV values for T-47D cells showed that the heated samples had slightly better CVs (4.07 versus 4.25). For T98G cells, samples stained with Triton-X 114 gave better G1_CV values than the samples stained only with Tergitol soap in solution A1 (4.16 versus 4.57).

In experiment 13, 0.25 ‰ and 0.375 ‰ Triton-X 114 in solution A6 with 22 and 15 minutes staining time were used for T98G cells, respectively. For T-47D cells, 0.25‰ Triton-X 114 in solution A6 with 15 minutes and 0.5‰ Triton-X 114 in solution A6 with 22 minutes were performed at room temperature (20 ^{0}C). After 13 experiments and considering both the photos and the G1_CV values in addition to cell lose during the experiments, it was found that:

- 0.5 ‰ Triton-X 114 in solution A with 22 minutes staining time for T-47D cell line
- 0.4 ‰ Triton-X 114 in solution A with 15 minutes staining time for T98G cell line

are best to use in the further experiments. But in last two experiments (17 and 18) for T98G LDRres cells 22 minutes was used instead of 15, so 22 minutes was chosen in the end.

Until now, it was shown how the method was developed and established. After this step, it was time to apply this method to the cells showing different characteristics. Mainly, it was comparison among:

- T98G cell line (T98G, T98G-P, and T98G LDRres)

- T-47D cell line (T-47D, T-47D-P, T-47D-P4, T-47D LDRres, F10, and F44)
- OR combination of these cells

to see the DNA content difference among them and the relevant cell cycle distributions.

It is necessary to state that when it is written 100 % of a cell type is mixed with 100 % of another type means: They are mixed without knowing the cell numbers compared to each other. On the other hand, when it is written 50 % versus 50 % or 10 % versus 90 % and so on, the cell numbers are known.

First T98G and T98G LDRres (Low Dose Rate resistance) cells will be analysed. T98G-P cells were not analysed with the Vindeløv method. But their DNA contents will be examined in the DNA-Protein method.

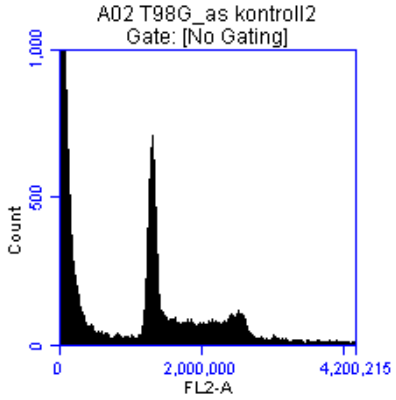


Figure 3.8(A): *T98G cells as control. G1_CV=3.88 ±0.01, experiment 14.*

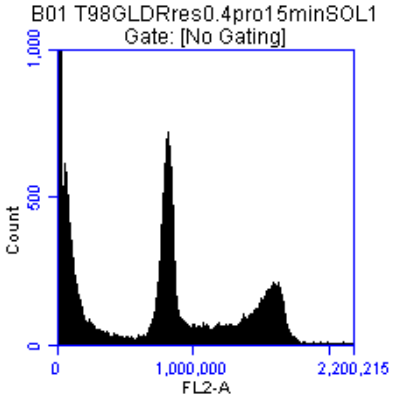


Figure 3.8(B): *T98G LDRres cells. G1_CV=5.20 ±0.01, experiment 14.*

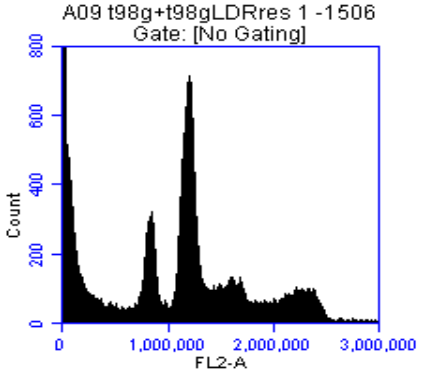


Figure 3.8(C): *50 % T98G + 50 % T98G LDRres cells with solution A8, 0.4 ‰ Tritonx-114 soap, 15 minutes staining at 20 ^{0}C. G1_CV =4.60 ± 0.01 for T98G LDRres and. G1_CV =4.60 ± 0.01 for T98G in experiment 16.*

The results show that the content of DNA of T98G LDRres cells was reduced (observe X-axis) compared to the control cells as shown in Figures 3-8(A-C). The mean values for G1 mean DNA content for 3 individually samples are given in Table 3.2.

Table3. 2: The G1 mean DNA content comparison of T98G and T98G LDRres for *mixed* cells. Mean values of individually prepared samples analysed in parallel.

Cell type	G1 mean	Percentage
MIXING of cells with each other (50 % T98G + 50 % T98G LDRres)		
T98G, 3 individual samples prepared in parallel in experiment 16	1323980.11 ± 28184.00	100 %
T98G LDRres, 3 individual samples prepared in parallel in experiment 16	926561.07 ± 12713.38	69.9 %

According to Table 3.2, it can be said that T98G LDRres cells have approximately **30 %** less DNA content than that of T98G cells.

As to the cell cycle distributions, which are shown in Table 3.3, both cell types have almost the same G1 phase while S phase of the control (T98G) cells are slightly higher than that of T98G LDRres. This slight increase in S phase in control cells results the same slight decrease in G2 phase in the control cells.

Table3. 3: The cell cycle distribution of T98G and T98G LDRres cells. Mean values of 3 individual samples with standard error. Mean values of individually prepared samples analysed in parallel.

Experiment 14			
Cell Type, 3 samples	G1 %	S%	G2%
T98G (Control) (mixing)	49,87 ± 2,74	38,29 ± 3,31	11,83 ± 1,32
T98G LDRres (mixing)	49,66 ± 2,81	30,03 ± 0,52	20,04 ± 2,33
Experiment 16			
Cell Type, 3 samples	G1 %	S%	G2%
T98G (Control) (no mixing)	50,75 ± 1,7	36,82 ± 1,49	12,44 ± 0,31
T98G LDRres (no mixing)	47,78 ± 10,17	33,20 ± 14,96	19,02 ± 4,57

Now T-47D cell line and its derivatives (F10, F44) will be examined. Here there is also no data about T-47D-P cells.

The DNA content of T-47D cells was investigated by mixing them with first T98G (in experiment 17) and later with T98G LDRres (in experiment 18). Also DNA content of F10 was compared with F44 by mixing them with each other in experiment 19

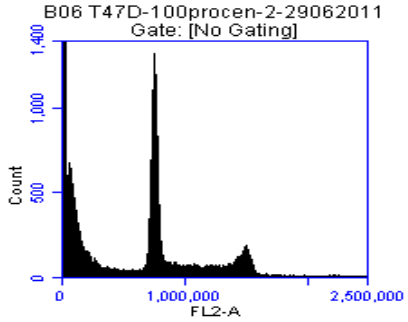


Figure 3.9(A): *T-47D, DNA histogram of T-47D with G1_CV =3.52, experiment 18.*

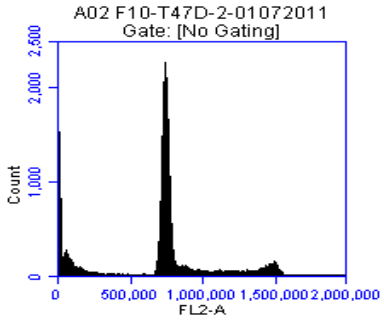


Figure 3.9(B): *F10, DNA histogram for 100 % F10 (T-47D) cells with G1_CV = 3.27 where average G1_CV =3.64 ± 0.21, experiment 19.*

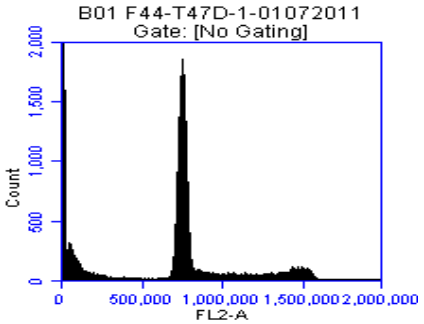


Figure 3.9(C): *F44, DNA histogram for 100 % F44 (T-47D) cells with G1_CV = 3.78 where average G1_CV =3.89 ± 0.13, experiment19.*

The G1 mean DNA contents of the cells in Figure 3.9(A-C) are given in Table 3.4.

Table3. 4: The G1 mean DNA content comparison of F10 and F44 cells in experiment 19. Mean values of individually prepared samples analysed in parallel.

Cell type	G1 mean	Percentage
MIXING of cells with each other (100 % F10 + 100 % F44)		
F10 (T-47D), 1 individual sample prepared in parallel in experiment 19	822385.05	100 %
F44 (T-47D), 1 individual sample prepared in parallel in experiment 19	822385.05	100 %

According to the figures in Table 3.4, it is possible to claim that there is no DNA content difference for F10 and F44 cells. This can also be seen in Figure 3.10(F).

The DNA histogram of T-47D, T98G LDRres, F10, and of F44 cells are shown in Figures 3.10(D-F).

The cell cycle distribution of T-47D cells and its derivatives are given in Table 3.5.

Table3. 5: The cell cycle distributions of T-47D cells and its derivatives. Mean values of individually prepared samples analysed in parallel.

Experiment 18			
Cell Type, 2 samples	G1 %	S%	G2%
T-47D	55,40 ± 0,54	31.13 ± 0,45	13,46 ± 0,09
Experiment 19			
Cell Type, 3 samples	G1 %	S%	G2%
F10 (T-47D)	70,19 ± 7,33	23,56 ± 8,99	6,24 ± 2,36
F44 (T-47D)	74,46 ± 5,59	19,49 ± 4,50	6,05 ± 1,11
Experiment 15			
Cell Type , 1 sample	G1 %	S%	G2%
F10 (T-47D)	58,54	29,68	11,78

It is useful to compare the controls with each other, T98G and T47D. *In experiment 17*, the DNA content of T-47D and T98G control cells were compared with each other by mixing them with 10 % versus 90%, 20 % versus 80 %, up to 50% versus 50%. The differences can be easily seen in Figures 3-10 (A-C).

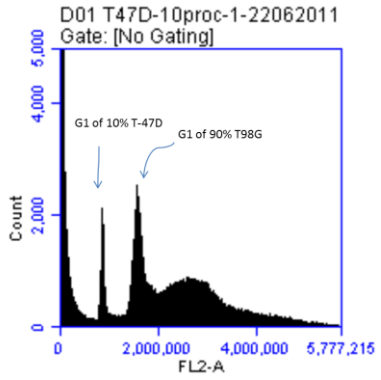


Figure 3.10(A): *DNA histograms for 10 % T-47D cells and 90 % T98G used in experiment 17.G1_CV of T-47D=5.82. G1_CV of T98G =5.73*

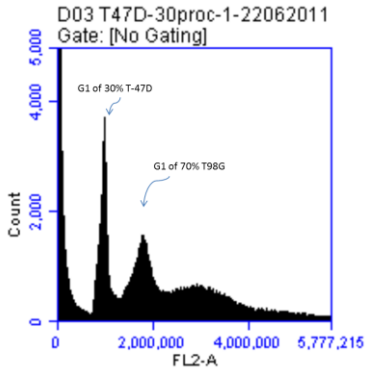


Figure 3.10(B): *DNA histogram for the T-47D cells used in experiment 17. G1_CV of T-47D=8.15. G1_CV of T98G =8.20*

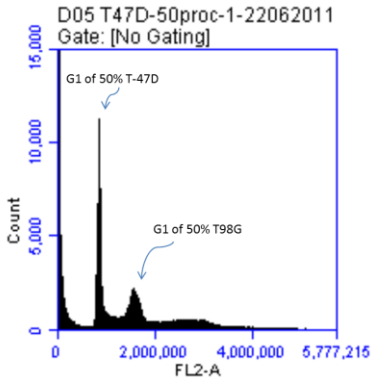


Figure 3.10(C): *DNA histogram for the T-47D cells used in experiment 17. G1_CV of T-47D=5.98. G1_CV of T98G =5.97.*

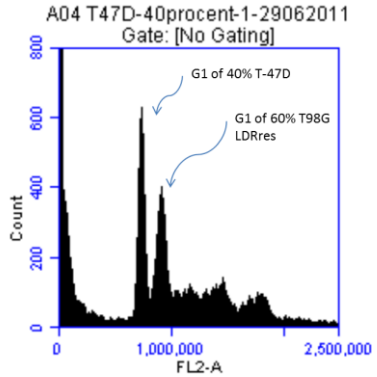


Figure 3.10(D): *40 % T-47D + 90 % T98G LDRres cells.*

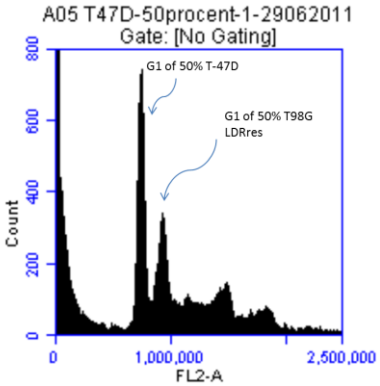


Figure 3.10(E): *50 % T-47D + 50 % T98G LDRres cells.*

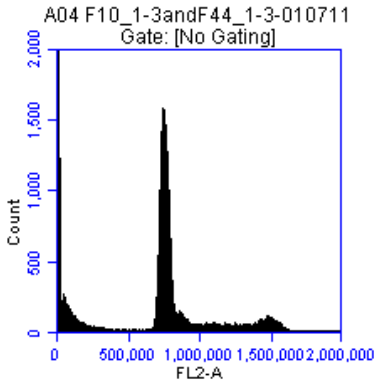


Figure 3.10(F): *DNA histogram for 100 % F10 (F-47D) 1-3 + 100 % F44 (T-47D) 1-3 cells with G1_CV = 4.31*

Table3. 6: The G1 mean DNA content comparison of T98G and T-47D cells by mixing. Mean values of individually prepared samples analysed in parallel.

Cell type	G1 mean	Percentage
MIXING of cells with each other (T-47 50 % + T98G 50 %)		
T98G, 2 individual samples prepared in parallel in experiment 17	1671682.45 ± 2138.63	100 %
T-47D, 2 individual samples prepared in parallel in experiment 17	894068.32 ± 10922.00	53.48 %

According to Table 3.6, the DNA content of T-47D cells is almost **47 %** less than that of T98G cells. This difference can easily be seen in Figures 3-10 (A-C).

Finally, the DNA content of T98G LDRres was compared with the DNA content of T-47D cells by mixing them in experiment 17. Due to software problem, the results could not be analysed. On the other hand, the DNA histograms show clearly that T-47D cells have less DNA content than that of T98G LDRres cells as shown in Figures 3.10(D-E).

3.2.2 The two-parametric DNA – Protein Method

Like in the Vindeløv method, in this part also the strategy was to divide it into two parts; 1- Develop and establish the method first, and later, 2- Apply the method to the cells which show different characteristics.

In this part, total 9 experiments were performed. The staining solutions were prepared according to the article [53], except one sample out of six gave bad results in the first experiment, i.e., no protein histogram. The DNA histogram and protein histogram for T98G cells are shown shown in Figures 3.11 (A-D).

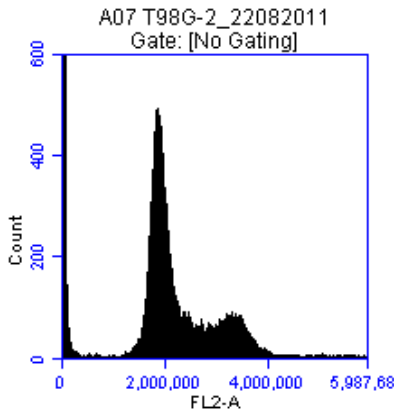


Figure 3.11(A): *T98G, DNA histogram for sample A07 in experiment 20.*

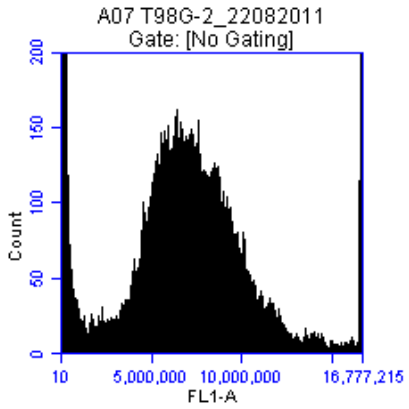


Figure 3.11(B): *T98G, protein (FL1-A) histogram for sample A07 in experiment 20, the desired protein histogram.*

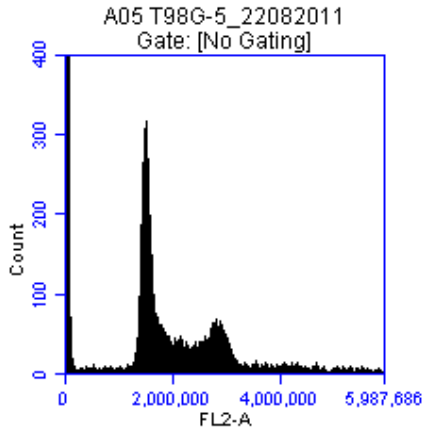


Figure 3.11(C): *T98G, DNA histogram for sample A05 in experiment 20.*

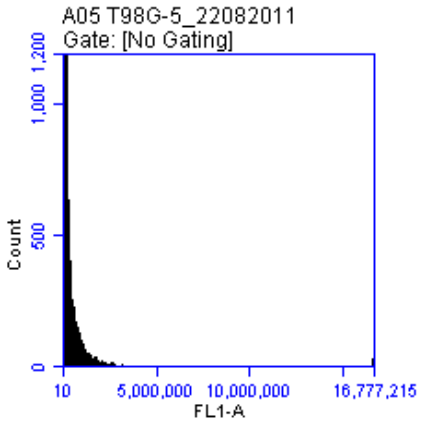


Figure 3.11(D): *T98G, DNA histogram for sample A05 in experiment 20, <u>not desired</u> protein histogram.*

This problem, no protein histogram, happened because of the unclear explanation of "Materials and Methods" section in the article. Total four experiments were performed according to the article. One of my supervisors (Erik Pettersen) found out the reason why no protein histogram was achieved after the four experiments. The reason was that the cells had to be stained separately in FITC solution and later in RNase solution. Before, they were stained in both of them at the same time which reduced the effect of FITC.

Before the solution was found out, the following was performed:

- The fixation of the cells both in 50 % and 70 % rectified spirit:

  This resulted slightly better G1_CV values for 70 % cells than that of 50 % (5.16 ± 0.35 versus 5.37 ± 0.26). In addition to this, it showed almost no difference in cell cycle distribution. More details about the cell cycle distribution are given in Table A.2.2 in Appendix A2.

- PI concentration was increased double, from17 µg/ml to 34 µg/ml, and it was used in the all experiments.
- Since there was problem with protein (FITC) histogram, FITC concentration was increased 10 times more, from 0.1 µg/ml to 1.0 µg/ml, because the FITC solution color was not green. It had the same color as water. This new concentration was used in one experiment and ignored later due to bad results.
- The good G1_CV results and DNA histograms with no protein histograms (shown in Figures 3.12(A-B)) indicated that the fixation (50 % ethanol) was very successful but something was very wrong with FITC.

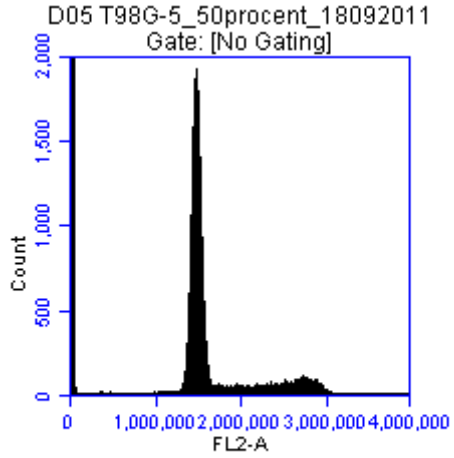


Figure 3.12(A): *T98G, DNA (FL2-A) histogram of 50 % Rectified spirit with G1_CV =3.72 ± 0.01 where average G1_CV = 3.79 ± 0.04 .*

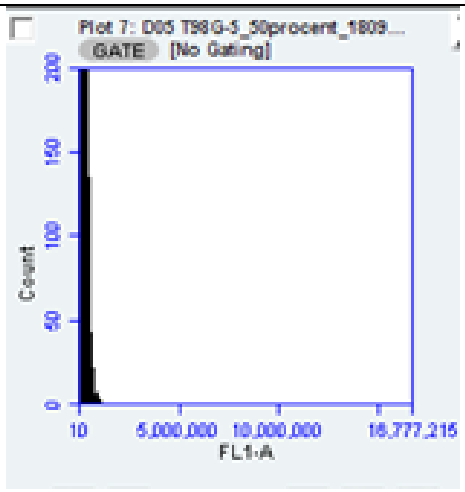


Figure 3.12(B): *T98G, protein (FL1-A) histogram for sample D05 with 50 % Rectified spirit.*

After finding out the solution for FITC problem, a new problem popped up, i.e., *spectral overlapping* between FITC and PI. This overlapping had to be compensated and it was solved by using FCS Express 3.0 software applying between 6.5 % and 8.5 % compensation to the corresponding dot plots. Appendix B explains how the compensation was performed using the software.

Now the DNA content and cell cycle distributions can be analysed as in the previous section. It is important to state that this method has not only nuclear DNA but also mitochondrial DNA. In the Vindeløv method, there was no mitochondrial DNA.

It is convenient to begin with T98G cell line and its derivatives. The DNA histogram for these cells after compensation is given in Figures 3.13(A-C).

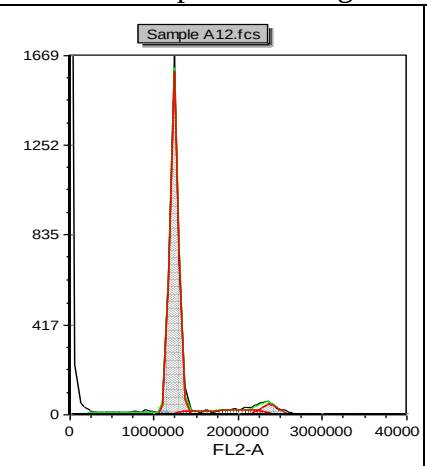


Figure 3.13(A): *T98G 100 % , sample A12 as control, after compensation. G1_CV=3.90 ±0.01, experiment 28.*

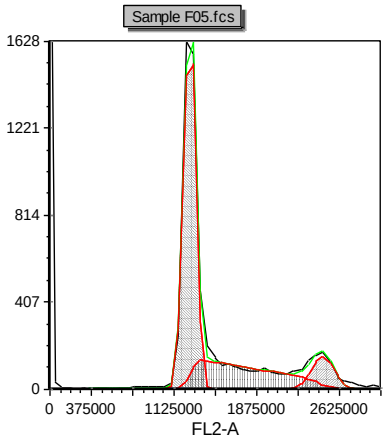


Figure 3.13(B): *T98G LDRres, sample F05 after compensation. G1_CV=3.93±0.01, experiment 25.*

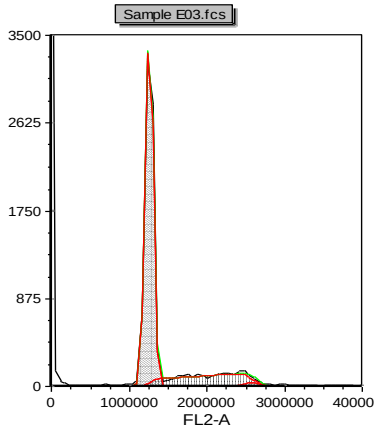


Figure 3.13(C): *T98G - P, sample F01 after compensation. G1_CV=3.49±0.01, experiment 24.*

Table3. 7: The G1 mean DNA content comparison of T98G and T98G LDRres cells for mixed cells. Mean values of individually prepared samples analysed in parallel.

Cell type	G1 mean	Percentage
MIXING of cells with each other (30% versus 70% , 40 % versus 60 %, and *50 % versus 50% for T98G LDRres and T98G cells, respectively)*		
T98G, 3 individual samples prepared in parallel in experiment 28	1317670.11 ± 4496.18	100 %
T98G LDRres, 3 individual samples prepared in parallel in experiment 28	959786.86 ± 2506.28	72.84 %

According to these figures in Table 3.7, T98G LDRres cells have approximately **27 %** less DNA content than that of T98G cells.

Table3. 8: The cell cycle distributions of T98G, T98G – P, and T98G LDRres cells. Mean values of individually prepared samples analysed in parallel.

Experiment 22			
Cell Type, 2 samples	G1 %	S%	G2%
T98G	69,92 ± 0,28	21,32 ± 1,90	8,75 ± 2,18
Experiment 23			
Cell Type, 4 samples	G1 %	S%	G2%
T98G	72,53 ± 0,36	23,35 ± 0,45	4,11 ± 0,18
Experiment 24			
Cell Type, 3 samples	G1 %	S%	G2%
T98G-P	72,11 ± 1,95	25,23 ± 1,69	2,65 ± 0,32
Experiment 27			
Cell Type, 4 samples	G1 %	S%	G2%
T98G LDRres	67,97 ± 0,48	21,40 ± 1,05	10,12 ± 0,82

According to Table 3.8, it is obvious that the cell cycle distributions are nearly the same for all the cell types.

As to T-47D cell line:

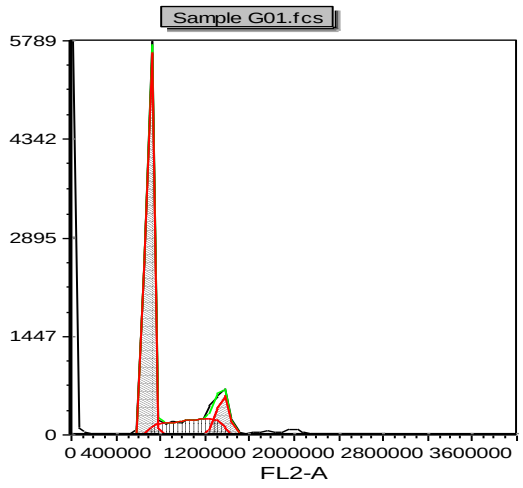


Figure 3.14(A): *T-47D, DNA (FL2-A) histogram with G1_CV =3.88 ± 0.01 where average G1_CV = 4.76 ± 0.52 .*

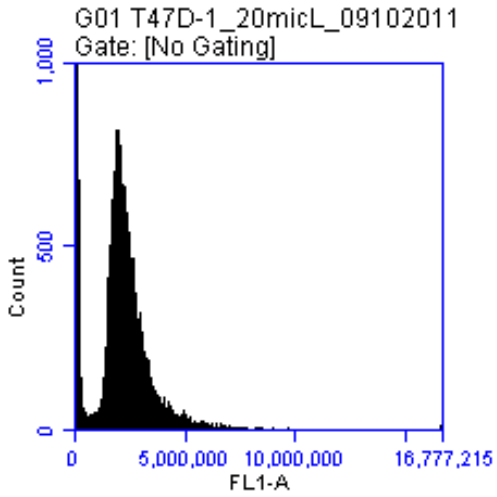


Figure 3.14(B): *T-47D, protein (FL1-A) histogram for sample GO1.*

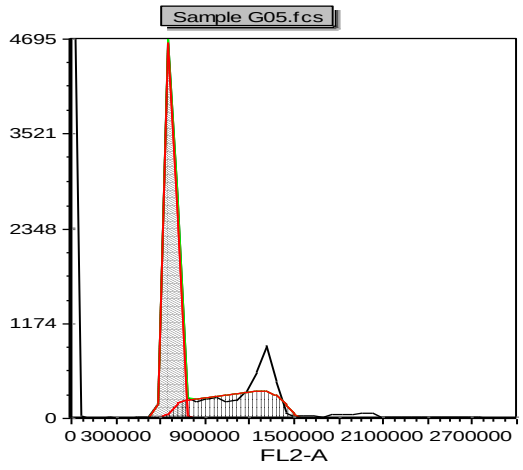


Figure 3.14(C): *T-47D - P, DNA (FL2-A) histogram with G1_CV =4.50 ± 0.01 where average G1_CV = 4.50 ± 0.10*

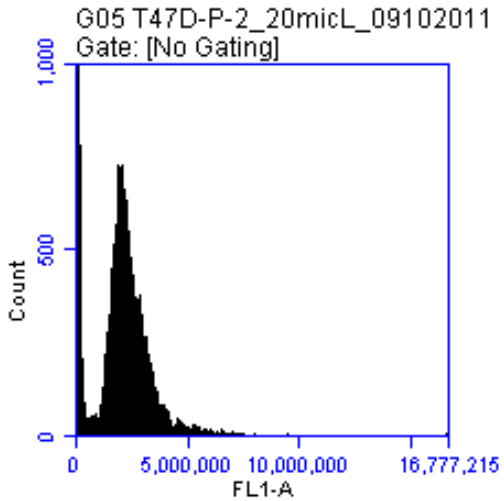


Figure 3.14(D): *T-47D-P, protein (FL1-A) histogram for sample GO5.*

Both DNA and protein histograms of T-47D and of T-47D-P cells are shown in Figures 3.14(A-D).

Table3. 9: The G1 mean DNA content comparison of T-47D, T-47D-P cells.

Cell type	G1 mean	Percentage
MIXING of cells with each other (100 % T-47D versus 100 % T-47D-P)		
T-47D (100 %) + T-47D-P (100 %), 1 sample from each individual samples prepared in parallel in experiment 26	744505.13	100 %

Taking into account both Figures 3.14(A-D) and Table 3.9, it is clear to see that the amount of protein for both types is the same and that both types have also the same DNA content.

So to the cell cycle distributions for these two types:

Table3. 10: The cell cycle distributions for T-47D and T-47D-P cells. Mean values of individually prepared samples analysed in parallel.

Experiment 26			
Cell Type, 3 samples each	G1 %	S%	G2%
T-47D	68,54 ± 1,21	19,50 ± 1,33	11,94 ± 1,26
T-47D –P	67,76 ± 0,38	23,78 ± 3,16	8,45 ± 3,45

The cell cycle distribution of T-47D-P cells does not appear to deviate from that of T-47D cells (Table.3.10).

It is necessary to take a look at the amounts of proteins for the cells which were analysed. Figures 3.15 (A-0) summarize the DNA-protein histograms before and after compensations and the corresponding protein histograms.

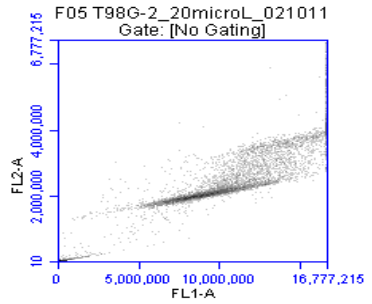


***Figure 3.15(A): T98G**, DNA-protein histogram with G1_CV =8.07 ± 0.01 BEFORE compensation.*

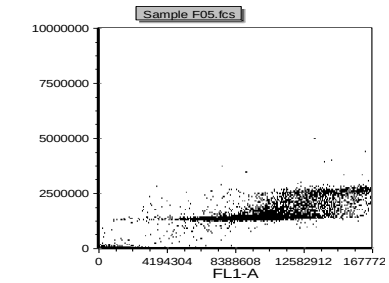


***Figure 3.15(B): T98G**, DNA-protein histogram with G1_CV =3.93 ± 0.01 AFTER 8.0 % compensation.*

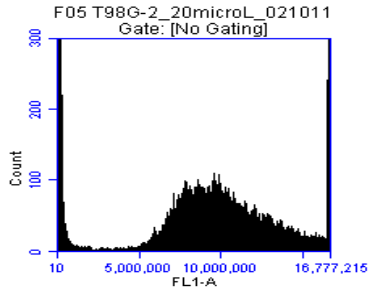


***Figure 3.15(C): T98G**, protein histogram.*

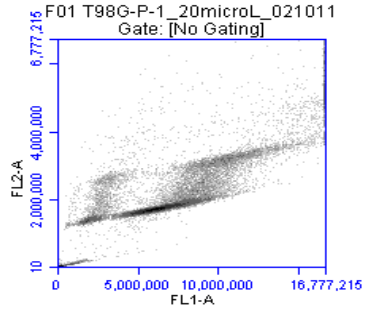


***Figure 3.15(D): T98G-P**, DNA-protein histogram with G1_CV =8.82 ± 0.01 BEFORE compensation.*

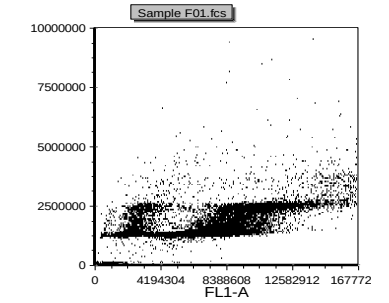


***Figure 3.15(E): T98G-P**, DNA-protein histogram with G1_CV =3.96 ± 0.01 AFTER 7.5% compensation.*

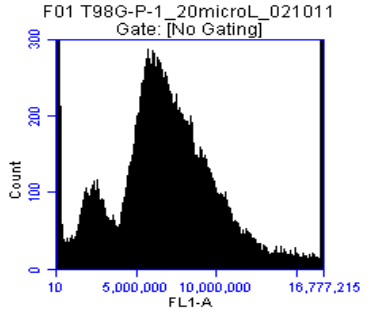


***Figure 3.15(F): T98G-P**, protein histogram. NOTE the second small peak on the left side.*

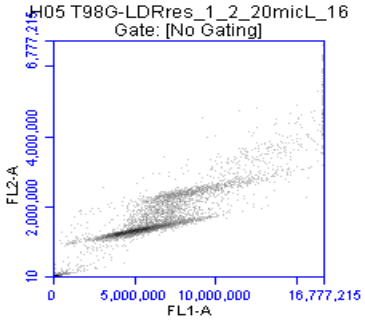


***Figure 3.15(G): T98G LDRres**, DNA-protein histogram with G1_CV =8.91 ± 0.01 BEFORE compensation.*

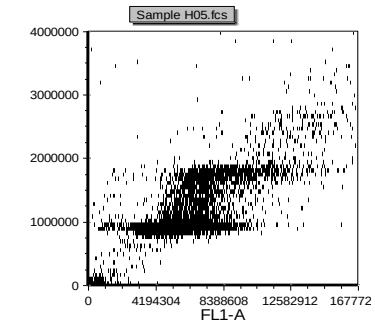


***Figure 3.15(H): T98G LDRres**, DNA-protein histogram with G1_CV =4.61 ± 0.01 AFTER 8% compensation.*

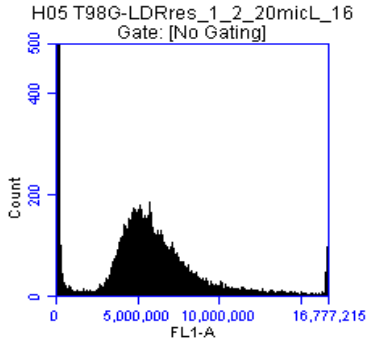


***Figure 3.15(I): T98G LDRres**, protein histogram.*

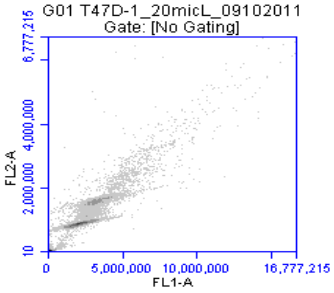


***Figure 3.15(J): T-47D**, DNA-protein histogram with G1_CV =5.94 ± 0.01 BEFORE compensation.*

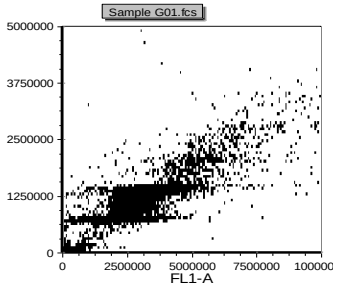


***Figure 3.15(K): T-47D**, DNA-protein histogram with G1_CV =3.88 ± 0.01 AFTER 8% compensation.*

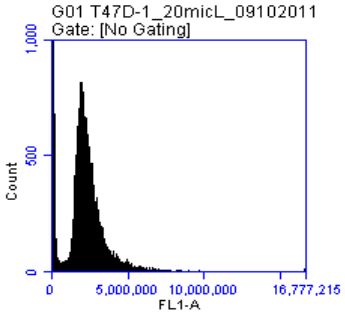


***Figure 3.15(L): T-47D**, protein histogram.*

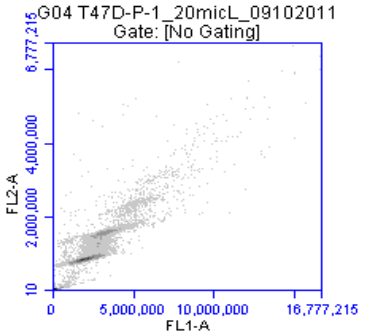


***Figure 3.15(M): T-47D-P**, DNA-protein histogram with G1_CV =6.25 ± 0.01 BEFORE compensation.*

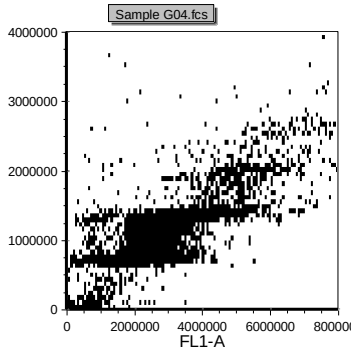


***Figure 3.15(N): T-47D-P**, DNA- protein histogram with G1_CV =4.28 ± 0.01 AFTER 8% compensation.*

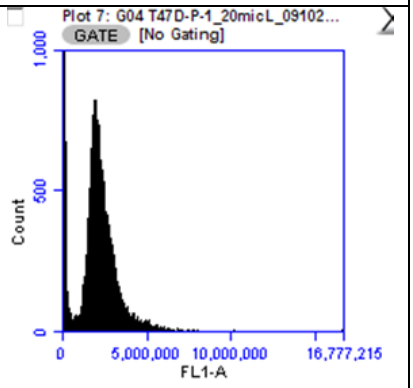


***Figure 3.15(O): T-47D-P**, protein histogram.*

According to Figures 3.15 (A-O), it is clear to say that both T-98G and T-98G LDRres have the same amount of proteins while T-47D and T-47D-P have also the same amount of proteins but less than that of T-98G and of T-98G LDRres.

So as to T98G-P cells, the most interesting result of this project can be seen in Figure 3.15 (D-F). The figures show that there is a sub-population in T-98G-P where it is clear to see that they have different amount of proteins. It became important to analyse whether they had also different DNA content or not. In the dotplot showing DNA amount versus protein amount the two subpopulations with different protein were gated(Figures 3.16 (A andC)) and the respective DNA histograms (Figures 3.16(B and D)) analysed. No difference in DNA content

was obseved for the two subpopulations. The difference in S % phases was nearly 25 % more for the subpopulation with less protein.

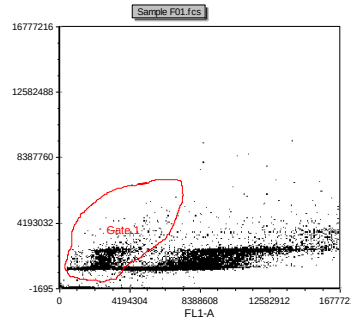


***Figure 3.16(A): T98G-P**, Protein content as function of DNA content AFTER 7.5% compensation, Gate 1 for the sub population with less amount of proteins.*

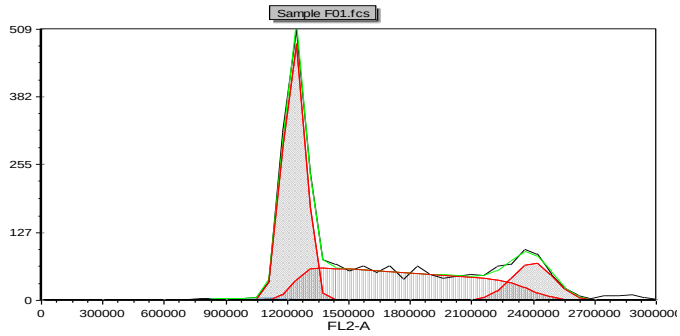


***Figure 3.16(B): T98G-P**, DNA histogram for Gate 1 with G1_CV =4.08 ± 0.01 and G1 mean = 1298827.50 AFTER 7.5% compensation. S % was 41.08*

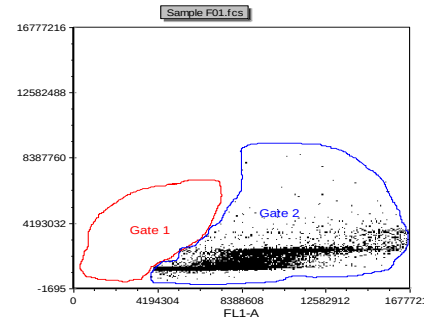


***Figure 3.16(C): T98G-P**, Protein content as function of DNA content AFTER 7.5% compensation, Gate 1 for the sub population with less amount of proteins.*

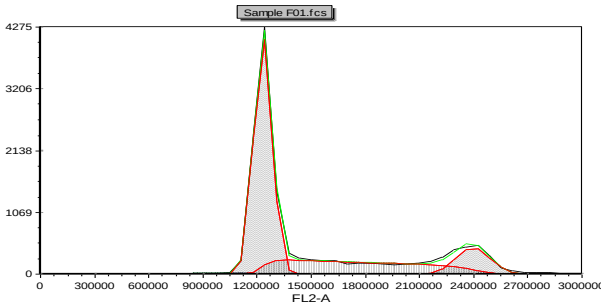


***Figure 3.16(D): T98G-P**, DNA histogram for Gate 2 with G1_CV =3.86 ± 0.01 and G1 mean = 1297300.36 AFTER 7.5% compensation. S% was 26.81.*

Table3. 11: DNA content comparison for T98G-P cells after relevant gating for three samples. Mean values of individually prepared samples analysed in parallel.

DNA content comparison for T98G-P cells after relevant gating			
Cell Type,	G1 MEAN for cells with SMALLER proteins	G1 MEAN for cells with LARGER proteins	Percentage
T98G-P, (1) sample FO1 in experiment 25, G1 peak channel number found manually	1298827.50	1297300.36	100 % versus 99.88%
T98G-P, (1) sample FO3 in experiment 25, G1 peak channel number found manually	1237275.79	1246752.92	99.24 % versus 100%
T98G-P, (1) sample E02 in experiment 24, G1 peak channel	1582569.90	1582569.90	100 % versus 100%

number found automatically by the software			

According to both Figures 3.16 (A-D) and Table 3.11, it is plausible to indicate that the sub-population (with smaller protein content) has the same amount of DNA as normal T98G-P cells.

Table 3. 12: List of figures and tables for good navigation in the result section after the methods were established.

The Vindeløv Method	
Figure number /Table name	*Explanation*
Figure 3.8 (A-C)	The DNA histograms for T98G, T98G LDRres, and 50 % mixing with each other
Table 3.2	The G1 mean DNA content comparison of T98G and T98G LDRres for *mixed* cells. Mean values of individually prepared samples analysed in parallel.
Table 3.3	The cell cycle distribution of T98G and T98G LDRres cells. Mean values of individually prepared samples analysed in parallel.
Figures 3.9 (A-C)	The DNA histograms of T-47D and its derivatives cells
Table 3.4	The G1 mean DNA content comparison of F10 and F44 cells in experiment 19. Mean values of individually prepared samples analysed in parallel.
Table 3.5	The cell cycle distributions of T-47D cells and its derivatives. Mean values of individually prepared samples analysed in parallel.
Figures 3.10 (A-C)	Comparing the DNA contents of T-47D and T98G , F10 and F44, and T-47D and T98G LDRres cells by mixing them with each other
Table 3.6	The G1 mean DNA content comparison of T98G and T-47D cells by mixing. Mean values of individually prepared samples analysed in parallel.
The two-parametric DNA –Protein Method	
Figures 3.11 (A-D)	The protein histogram problem shown by help of DNA and protein histograms of T98G cells
Figures 3.12 (A-B)	Example for good fixation with 50 % ethanol and the protein problem shown by help of T98G cells
Figures 3.13 (A-C)	The DNA histograms after compensation for T98G, T98G LDRres, and T98G-P cells
Table 3.7	The G1 mean DNA content comparison of T98G and T98G LDRres cells both for mixed cells. Mean values of individually prepared samples analysed in parallel.
Table 3.8	The cell cycle distributions of T98G, T98G – P, and T98G LDRres cells. Mean values of individually prepared samples analysed in parallel.
Figures 3.14 (A-D)	Both DNA and protein histograms of T-47D and of T-47D-P cells
Table 3.9	The G1 mean DNA content comparison of T-47D, T-47D-P cells.
Table 3.10	The cell cycle distributions for T-47D and T-47D-P cells
Figures 3.15 (A-O)	The DNA-protein histograms before and after compensations and the corresponding protein histograms for all the available cell types
Figures 3.16 (A-D)	The DNA-protein compensated dot plots and corresponding DNA histograms after relevant gating of T98G-P and its sub population
Table 3.11	DNA content comparison for T98G-P cells after relevant gating for three samples. Mean values of individually prepared samples analysed in parallel.
Table 3.12	List of figures and tables for good navigation in the result section after the methods were established

4 DISCUSSION

The goals of this master project were to develop and establish the methods:

1- The Vindeløv method which analyses only nuclear DNA content of the cells by ignoring the mitochondrial DNA with the help of cell nuclei isolation and relevant staining solutions. After obtaining nuclei from whole cells, the nuclei are treated with RNase and stained in PI solution before flow cytometric measurements.

2- The DNA- Protein method which can simultaneously measure both the protein content and the DNA content of whole cells. After relevant staining solutions, the cells are ready for flow cytometric measurements.

It is better to discuss them one by one.

4.1 The Vindeløv Method

In section 2.5.6 it was discussed what Vindeløv and his colleagues mainly did and how they developed the methods they needed.

In this project, the goal was to develop and establish the Vindeløv method as described in 2.5.6 at the Biophysics Cell Laboratory at the University of Oslo. The first problem was to find a replacement for the soap they used in their staining solution A because it was not produced any more. To replace the soap, i.e., solution A, three different types of soaps with altered concentrations were tried: Tergitol, SDS, and Triton – X 114. In addition to this, the staining times in solution A for these cells were also altered. Furthermore the effect of temperate (20 ^{0}C versus 37 ^{0}C) on the cells were examined. PBS (1X) was exchanged with 0.9 % NaCl to clean the cells. The heated samples (from 20 ^{0}C to 37 ^{0}C) had slightly better G1_CVs (4.05 ± 0.01 versus 5.05 ± 0.01) for T-47D cells (Figure 3.6(A-D)). After the method was established, room temperature (20 ^{0}C) was chosen.

Trying these different combinations to obtain nuclei from whole cells demanded much time, carefulness, and quickness while the cells were supposed to be stained for a certain amount of time. Sometimes one person was not enough to do many things in a short time with a good accuracy. Therefore, once I got help from my supervisor Nina Edin and Joe Alexander Sandvik to try many combinations in a short time. This help contributed a great deal to find the correct soap, its concentration, and the staining time in solution A. While trying to find the correct combination, it was important to use the resources we had carefully. Therefore the optimal solution A developed for both T-47D and T98G cell lines contains both Tergitol and Triton- X 114 soaps. One cannot exclude that even one single type of soap could do the same work, but in our hands this particular mix worked well.

As it was mentioned in section 2.5.4.4, the quality of a DNA histogram depends on *sample preparation, instrument alignment, staining procedure*. Sample preparation was important and it was the most difficult part because it takes a lot of time and it is very easy to do something wrong by mistake which will affect the results badly. A microscope during sample

preparation was used to observe cells and nuclei. Instrument alignment was checked each time before FCMs by using two types of fluorescent beads. One type of bead solutions gives 6 different peaks for one laser in the flow cytometer while the other bead solution type gives 8 peaks for the other laser in the flow cytometer. These routine bead measurements were necessary to check the linearity of the flow cytometer. Staining procedure was much more important in the DNA-Protein method compared to the Vindeløv method.

The quality of DNA histograms in this part of the project can be seen in Figures 4.1 (A-B).

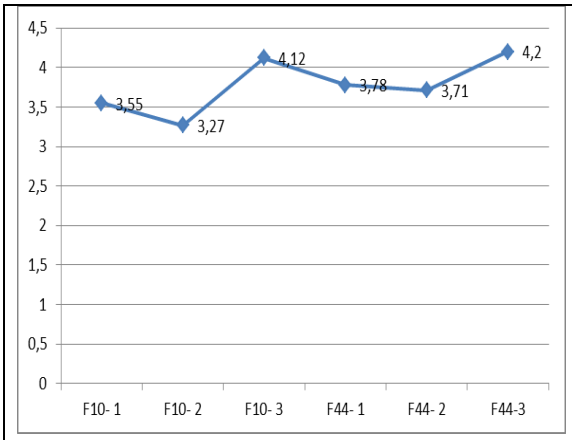


Figure 4.1(A): *G1_CV values of 100 % F10 (T-47D) cells with 3 samples where average G1_CV =3.64 ± 0.21 and of 100 % F144 (T-47D) cells with 3 samples where average G1_CV =3.89 ± 0.12.*

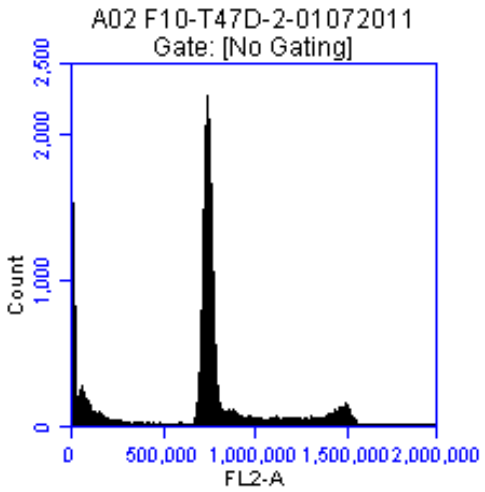


Figure 4.1(B): *DNA histogram for 100 % F10 (T-47D) cells with G1_CV = 3.27 where average G1_CV =3.64 ± 0.21.*

Comparing these G1_CVs with some G1_CVs in Vindeløv's article [41] page 319 shown in Table 4.1, gives confident to claim that this method in this project is as reliable as the Vindeløv method itself.

Table 4.1: Some G1_CV values from Vindeløv's article [41], page 319, Table 1.

cv(G1), 0 month Storage time	3.83, 3.53, 3.56, 3,50, 3.74, 3.63 Mean= 3.63, SD=0.14
cv(G1), 0.5 months Storage time	4.38, 4.18, 4.29, 4.19, 4.36, 4.28, Mean= 4.28, SD=0.09

In this project when the DNA histograms were analysed, no gating was performed to include all the raw data into account. Moreover, there was no need for gating using just one dye, PI. DNA histograms were analysed with FCS Express 3.0 software where six models were utilized to examine the data as shown in Figures 4.2(A-C). Applying different models to the histogram will vary the results slightly. Like Apsmodal, I also chose model 6 = *CVs Fixed* during the whole project because of its reliability.

Model	Dip %G2	Dip %S	Chis
SL S0	6.86	20.09	1.47
Sliced Bkgd	6.84	20.10	1.47
G2/G1 Fixed	1.40	25.08	2.10
Aggregates	0.00	26.24	1.43
S Order = 1	4.28	22.71	1.35
CVs Fixed	3.66	24.16	1.42

Figure 4.2(A): *The Models for DNA histogram Analysis (results from a sample) in FCS Express 3.0 Software*

Figure 4.2(B): *The Models choosing tool in FCS Express 3.0 Software*

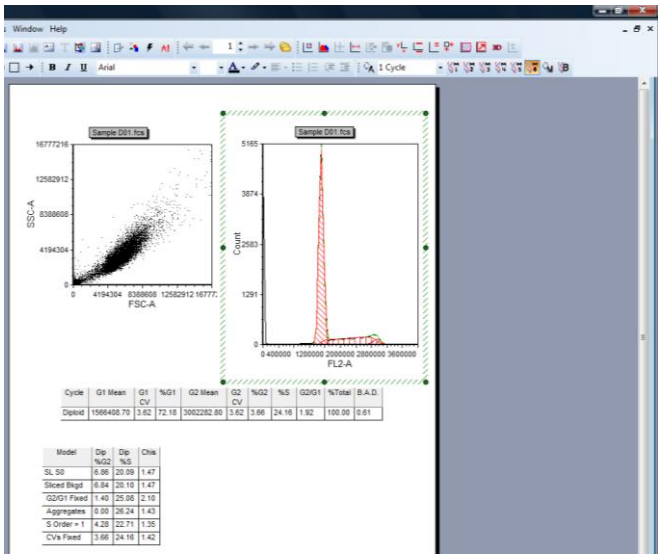


Figure 4.2(C): *A print screen view of one sample being analysed in FCS Express 3.0 software.*

Another important thing to mention is that no trout – and chicken red blood cells were used in this project as internal standards. Since determination of nuclear DNA content by flow cytometry is a relative method, the DNA content was instead compared by mixing the cells.

The most interesting result found by analysing the different types of cells after the Vindeløv method was established was the almost 30 % decrease (Table 3.2) in DNA content of T98G LDRres cells compared to T98G cells. This decrease 27 % (Table 3.7) (3 % less than the Vindeløv method) was also observed in the two-parametric DNA-Protein method.

The reason why T98G LDRres had less DNA content is interesting to discuss:
As mentioned in previous sections, T98G LDRres Low Dose Rate resistant cells are the cells which grown in [^{3}H]-medium with a specific activity of 1.67 µCi/ml for 10 months. These cells nearly died out but a small subpopulation survived and proliferated. The data showed that the resistant subpopulation had a changed DNA-ploidity. As seen in Table 4.1 there was also a slight change in the cell cycle distribution.

Table 4.1: The Cell cycle distributions for both T98G and T98G LDRres cells

Experiment 14			
Cell Type, 3 samples	G1 %	S%	G2%
T98G (Control)	49,87 ± 2,74	38,29 ± 3,31	11,83 ± 1,32
T98G LDRres	49,66 ± 2,81	30,03 ± 0,52	20,04 ± 2,33
Experiment 16			
Cell Type, 3 samples	G1 %	S%	G2%
T98G (Control)	50,75 ± 1,7	36,82 ± 1,49	12,44 ± 0,31
T98G LDRres	47,78 ± 10,17	33,20 ± 14,96	19,02 ± 4,57

The results of experiment 14 seem more reliable compared to the results of experiment 16 especially for T98G LDRres where the standard errors of the means are worse than that of in experiment 14. The figures indicate that G1% is the same for both types. S% of T98G is

nearly 8 % higher than that of T98G LDRres where this 8 % increase is shown as decrease in G2 % of T98G compared to S % of T98G LDRres. It seems that it was easier for T98G LDRres cells to pass through S compare to the T98G cells and thereafter they were blocked in G2 by a check-point which was triggered to be active because of the low –dose rate irradiation.

In order to exclude the possibility that the resistant subpopulation of T98G LDRres was due to contamination with T-47D cells, these two cell were mixed. The two distinct G1 peaks supported the morphological observations that T98G LDRres were not T-47D cells. T-47D cells had nearly half the DNA content compared to that of T98G cells (Table 3.6). Comparing result of Table 3.6 with the results of Table 2.1 where the cell average nucleus diameter for T-47D and T98G were nearly the same, 11.00 ± 1 or 11.11 ± 0.182, respectively according to the study of Aspmodal. One can say that the reason of the DNA content difference could lead us to have an idea about their DNA content densities. The DNA content in T98G cells could be packed more densely.

The treatment of growing in 4% hypoxia and in [^{3}H]-medium with a specific activity of 1.67 μCi/ml for 35 days did not affect the DNA content of T-47D cells of the F44 freezing protocol compared to that of T-47D cells of the F10 freezing protocol which had almost the same background without hypoxia (Table 3.4). This could mean that 4% hypoxia had no effect on F44 (T-47D) cells when the treatment was combined with a low dose rate irradiation.

4.2 The two-parametric DNA – Protein Method

This method which was developed and established based on the study of Øystein W. Rønning and Tore Lindmo in Oslo in 1983[51]. They investigated whether human NHIK 3025 cells are dependent upon a net increase in cellular protein content in order to traverse G1 and S. What they found was that the net accumulation of protein did not seem to be a prerequisite for traverse through G1 and S, that is, DNA replication may be dissociated from the general growth of cell mass. Their method enabled them to observe the increase in DNA and protein content which was studied by means of two-parameter flow cytometry using populations of cells synchronized by mitotic selection.

When this method was attempted to be established in the present study, the first problem was that no *protein histogram was recorded* using the FITC dye, expect only one sample out of six in the first experiment. Three more experiments were repeated by applying slight changes such as increasing the FITC concentration 10 times more. Still no protein histogram was obtained. On the other hand, the DNA histograms indicated how successful the fixation and sample preparation were. Both the DNA histogram and protein histogram (the problem) can be seen in Figures 4.3(A-B).

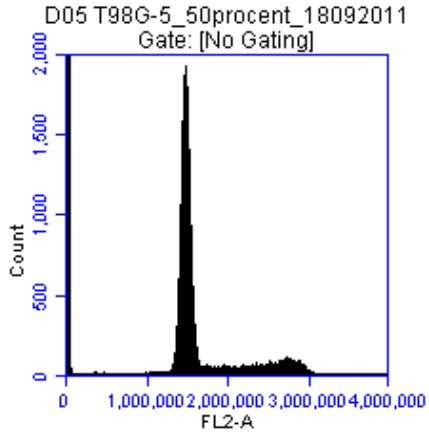


Figure 4.3(A): *T98G, DNA (FL2-A) histogram of 50 % Rectified spirit with G1_CV =3.72 ± 0.01 where average G1_CV = 3.79 ± 0.04 .*

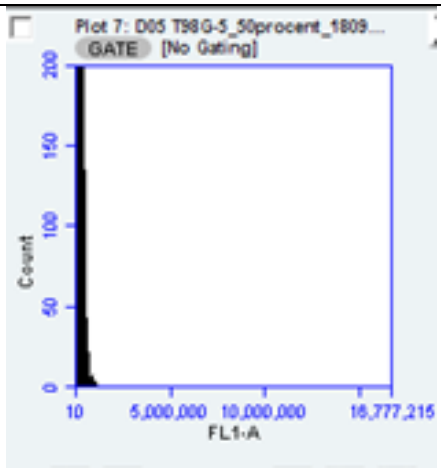


Figure 4.3(B): *T98G, protein (FL1-A) histogram for sample D05 with 50 % Rectified spirit (Ethanol).*

The problem was that there was an error in the methodology description in the paper of Rønning and Lindmo which was discovered by one of my supervisors, Erik Pettersen. The cells had to be stained separately in FITC solution and later in RNase solution. In the first experiments, they were stained in both of them at the same time which reduced the effect of FITC. This problem could be actually solved earlier if other relevant articles had been reviewed.

Increasing FITC concentration from 0.1µg/ml to 1 µg/ml gave unreliable results (Figures A.2.6(C-D)). Therefore in all the next experiments 0.1µg/ml was used.

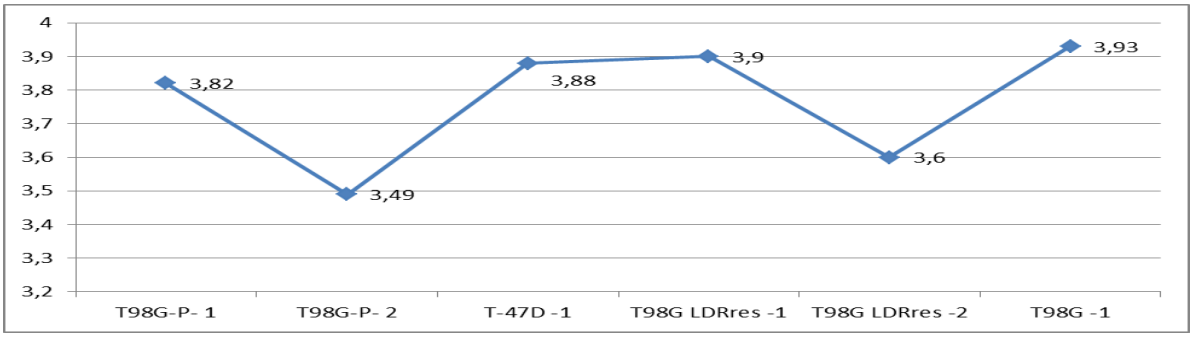


Figure 4.4: *Some G1_CV values of T98G-P, T-47D, T98G LDRres, and T98G cells taken from different experiments.*

The G1_CV values shown in Figure 4.4 indicate the quality of the DNA histograms obtained by this method. To improve the quality, during staining procedure the fixed cells were overnight in the cold and enough time was given to the RNase to remove all double –stranded RNA. As in the previous section, model 6 = *CVs Fixed* and no *gating* were utilized.

The second problem, *spectral overlapping* between FITC and PI, was compensated by FCS Express 3.0 software. The software could not manage to detect correct G1 peak channel numbers so it was fixed/estimated manually. This estimation affected G1_CVs ± 0.01 which is shown in Appendix H. The effect of compensation rates on G1_CVs and cell cycle distributions are shown in Table 4.3.

Table 4.3: The effect of compensation on G1_CVs and cell cycle distributions

BEFORE Compensation					
Cell type	*G1_CV*	*G1 Mean*	*G1%*	*S%*	*G2%*
T98G LDRres-1, Compensation 8.0%	8.91	1379139.58	65.67	31.75	2.58
T98G LDRres-2, Compensation 8.0%	8.40	1382462.26	61.72	27.75	10.53
T98G LDRres-3, Compensation 8.5 %	8.82	1546597.17	63.97	29.50	6.52
AFTER Compensation					
Cell type	*G1_CV*	*G1 Mean*	*G1%*	*S%*	*G2%*
T98G LDRres-1, Compensation 8.0 %	4.61	970428.92	69.63	19.31	11.06
T98G LDRres-2, Compensation 8.0 %	4.71	9882266.78	67.37	20.61	12.02
T98G LDRres-3, Compensation 8.5%	5.38	1110329.50	67.64	20.76	11.60

According to the Table 4.3, after the compensation rates on three samples of T98G LDRres in the same experiment, the followings can be claimed:

- The G1_CVs decreased (better results) almost 50 %.
- Mean value of the G1-peak (i.e., DNA content) (no mixing but same experiment) decreased almost 30 %.
- G1 % increased almost 5 %.
- S % decreased almost 10 %.
- G2 % increased almost 5 %.
- Two same compensation rates (8.0%) can still give different results which is normal because compensation varies from sample to sample.

Taking into account all these points, one can state that the DNA content found from G1 means and the cell cycle distributions depend strongly on the compensation rates. Therefore slight changes both in mean value of the G1-peaks (DNA content) and cell cycle distributions do not necessarily mean that they do really vary from each other. They could indeed contain the same amount of DNA and have similar cell cycle distribution values.
The most interesting result of the study invloving two-parametric recordings of DNA and protein was the sub-population detected in T98G-P cells having different amounts of protein (Figures 3.15(D-F)). The T-47D-P cells were also investigated but no such sub population was observed(Figures 3.15(M-O)). This sub-population of T98G –P cells had approximately half the protein content (an estimation according to Figure 3.15(F)) compared to that of wild-type, T98G-P cells. Both the sub-population and the wild- type had the same DNA content(Table 3.8).

What the reason could be for this extra-ordinary finding is very interesting to discuss but also very difficult to find the exact answer. This should be investigated with more experiments especially by finding a way to separate these two subpopulations from each other and culture them separately for further investigations. It would for example be interesting to compare radiosensitivity.

It is interesting that both the change in DNA – plodity after long term low dose rate irradiation and the emergance of a subpopulation with different protein content after low dose rate priming for 1 hour were observed in T98G cells, but not in T-47D cells. T98G cells are derived from a patient with glioblastoma multiforma. These patients have a poor prognosis because this cancer is aggressive and resistant to treatment. The findings in this study confirm that these cells change and adapt in response to radiation which may in part explain why these tumours are so difficult to treat.

Fixation of T98G cells either with 50 % or 70 % rectified spirit resulted in no difference in the cell cycle distributions (Table A.2.2 in Appendix A.2). This means fixation either in 50 % or 70 % did not affect the cell cycle distribution of T98G cells.

Several problems had to be solved in this project: To find a detergent which was suitable for the Vindeløv method, no protein histograms for a while, the risk of mixing cells with each other from growing in the medium until FCMs, the risk of that the cells could get infection. Another problem was the number of cells during FCMs. In general, this problem did not show itself but in some experiments due to low cell numbers it took up to 30 minutes to run a sample. If the result of that sample was very important then the only solution was to be patient

and wait. This long time sample running had the risk of ending up with no fluid in the sample tube which creates bad results for that sample result. Fortunately this never happened this in this project except one sample which was used to count the number of a cell type which would be mixed with another cell type.

Finally, one can say that the two-parametric DNA –protein method worked very well. Even if in experiment 26 some doublets on the DNA- protien dot plot were observed, this number is negligible compared to the single cell population on the DNA histogram shown in Figure 4.5.

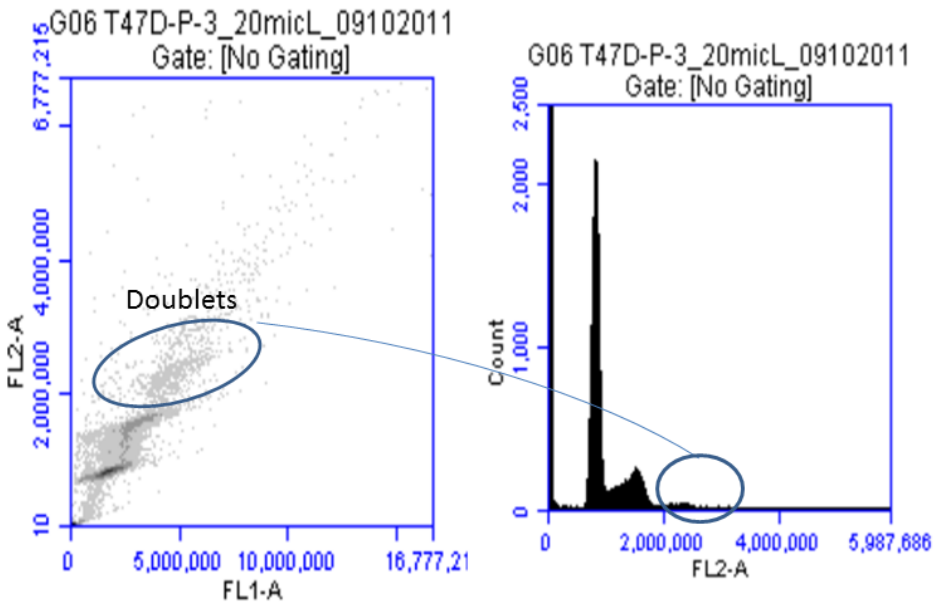


Figure 4.5: *Position of doublets on DNA- protein dot plot and DNA histogram.*

5 CONCLUSION

The main findings are:

- *The Vindeløv Method*

  - The Vindeløv method was successfully established and tested.
  - The heated samples (from 20 ^{0}C to 37 ^{0}C) had slightly better G1_CVs (4.05 ± 0.01 versus 5.05 ± 0.01) for T98G-P cells.
  - T98G LDRres cells had approximately 30 % less DNA content compare to T98G control cells.
  - The treatment of growing in 4% hypoxia and in [^{3}H]-medium with a specific activity of 1.67 μCi/ml for 35 days did not affect the DNA content of T-47D cells of the F44 freezing protocol compared to that of T-47D cells of the F10 freezing protocol which had almost the same background without hypoxia.
  - T-47D cells had nearly half the DNA content compared to that of T98G cells.

- *The two – parametric DNA -Protein Method*

  - The two-parametric DNA -Protein method was successfully established and tested.
  - Fixation of T98G cells either with 50 % or 70 % rectified spirit resulted in no difference in the cell cycle distribution of T98G cells.
  - Increasing FITC concentration from 0.1μg/ml to 1 μg/ml gave unreliable results.
  - T98G LDRres cells here had also approximately 27 % less DNA content compare to T98G control cells.
  - A sub-population of T98G-P cells was observed which had the same DNA content but less amount of protein.

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I wish to thank Joe Alexander Sandvik, Stine Christoffersen, Ida Aspmodal for their all kinds of help, especially for preparing the cells for my experiments. I thank also all the people at the Biophysics group for creating a friendly environment.

I want also to thank Eli Olaug Hole for her help during the whole master programme.

Blindern, May 2012

Celal Ceyhan

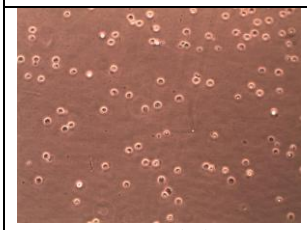
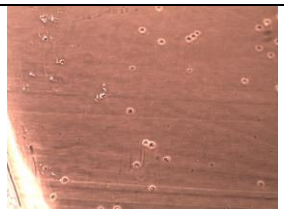
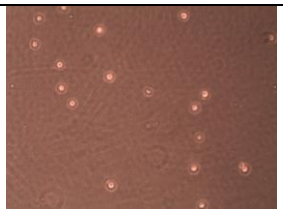
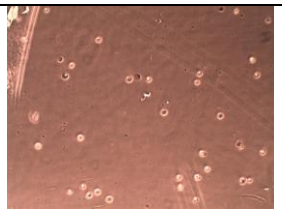
8 APPENDICES

Appendix A: Detail experimental results

Appendix A.1: The Vindeløv Method

Stock Solution1	
• Trisodium citrate 2 H_2O • Tergitol (soap) • Sperminetetrahydrochloride • Tris (hydroxymethyl)-aminomethane • Total volume (milliQ water) • PH adjusted to	2000 mg (3.4 mM) 2 ml (0.1 % v/v) 1044 mg (1.5 mM) 121 mg (0.5 mM) 2000 ml 7.6
Stock Solution2	
• Trisodium citrate 2 H2O • No soap • Sperminetetrahydrochloride • Tris (hydroxymethyl)-aminomethane • Total volume (milliQ water) • PH adjusted to	1000 mg (3.4 mM) 522 mg (1.5 mM) 61.5 mg (0.5 mM) 1000 ml 7.6
Solution A1	
• Stock Solution1 • Trypin (Sigma, T0134) • PH adjusted to	500 ml 15 mg 7.6
Solution A2	
• Stock Solution1 • Trypin (Sigma, T0134) • Tergitol soap • PH adjusted to	100 ml 3 mg 4 ml (40 % v/v) 7.6
Solution A3	
• Stock Solution2 • Trypin (Sigma, T0134) • No soap • PH adjusted to	50 ml 1.5 mg 7.6
Solution A4	
• Solution A3 • Triton-X soap • PH adjusted to	15 ml 15 µl (0.001 % v/v) 7.6
Solution A5	
• Solution A1 • SDS soap	- 0.1 % Tergitol versus 0.001 % SDS Or 1% Tergitol versus 0.001% SDS

Solution A6	
• Stock Solution1 • Trypin (Sigma, T0134) • Triton-X 114 soap • PH adjusted to	100 ml 3 mg 50 µl (0.5 % v/v) 7.6
Solution A7	
• Stock Solution1 • Trypin (Sigma, T0134) • Triton-X 114 soap • PH adjusted to	100 ml 3 mg 100 µl (1 % v/v) 7.6
Solution A8	
• Stock Solution1 • Trypin (Sigma, T0134) • Triton-X 114 soap • PH adjusted to	100 ml 3 mg 100 µl (0.4 % v/v) 7.6

EXPERIMENT 1				
Date	08.02.2011			
Used cell types and solutions with staining time				
Cell Type	T-47D	T-47D-P	T98G	T98G-P
Stock Solution	Stock Solution1			
Solution A	Solution A1, 10 minutes			
Solution B	Solution B, 10 minutes			
Solution C	Solution C, minimum 20 minutes			
Citrate Buffer	Citrate Buffer, 30 minutes			
PBS	PBS			
The Results of the Experiment				
FCM	Not performed (no staining in solution C)			
Photos				
 **Figure A.1.1(A):** *T47D*	 **Figure A.1.1(B):** *T47D-P*	 **Figure A.1.1(C):** *T98G*	 **Figure A.1.1(D):** *T98G-P*	

EXPERIMENT 2		
Date	23.02.2011	
Used cell types and solutions with staining time		
Cell Type	T-47D	T98G
Stock Solution	Stock Solution1	
Solution A	Solution A2, 10 minutes	
Solution B	Solution B, 10 minutes	
Solution C	Solution C, minimum 20 minutes	
Citrate Buffer	Citrate Buffer, 30 minutes	
PBS	PBS	
The Results of the Experiment		
FCM	Not performed (no staining in solution C)	
Photos		

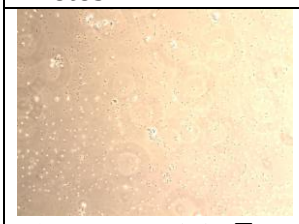


Figure A.1.2(A): *T-47D*

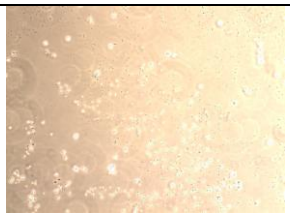


Figure A.1.2(B): *T-47D*

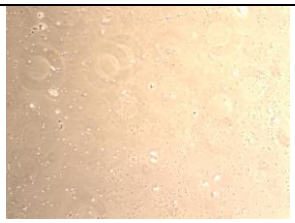


Figure A.1.2(C): *T98G*

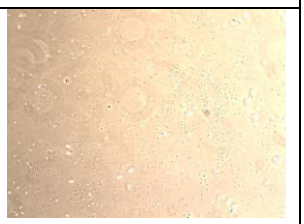


Figure A.1.2(D): *T98G*

EXPERIMENT 3	
Date	02.03.2011
Used cell types and solutions with staining time	
Cell Type	unknown
Stock Solution	Stock Solution2
Solution A	Solution A3 + Solution A4, 10 minutes
Solution B	Solution B, 10 minutes
Solution C	Solution C, minimum 20 minutes
Citrate Buffer	Citrate Buffer, 30 minutes
PBS	PBS
The Results of the Experiment	
FCM	Not performed (no staining in solution C)
Photos	No photos

EXPERIMENT 4		
Date	16.03.2011	EVENT(Cell) number : 20 000
Used cell types and solutions with staining time		
Cell Type	T-47D	T98G
Stock Solution	Stock Solution1	
Solution A	Solution A1- 10 mins, Solution A4- 10 mins, Solution A1 + A4 - 10 mins, Solution A1 + A5- 10 mins, Solution A4 + A5 - 10 mins.	
Solution B	Solution B, 10 minutes	
Solution C	Solution C, minimum 20 minutes	
Citrate Buffer	Citrate Buffer, 30 minutes	
NaCl	0.9% NaCl	
The Results of the Experiment		
FCM	The results were not good enough to be considered. Therefore only one of the samples will be showed here:	

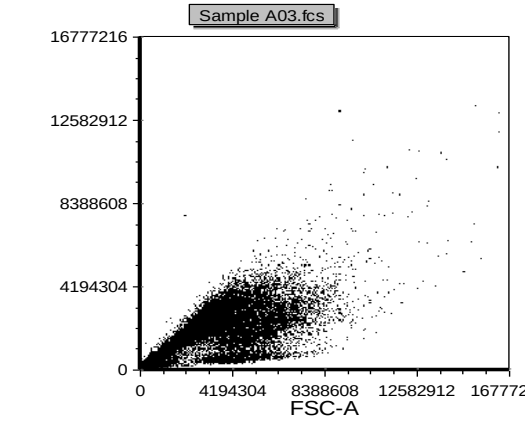


Figure A.1.3(A): *The dot plot for sample A03.*

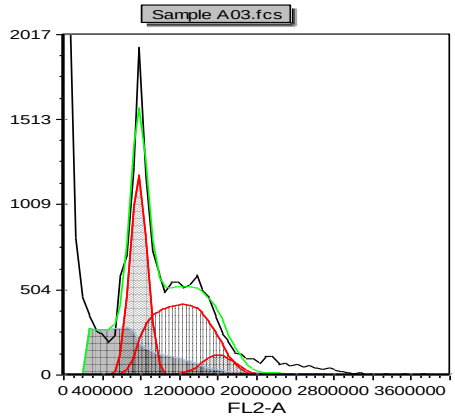
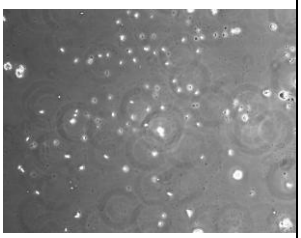
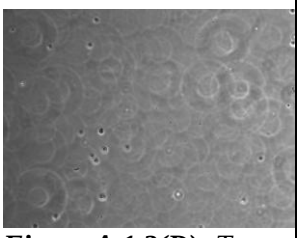
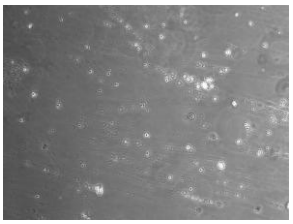
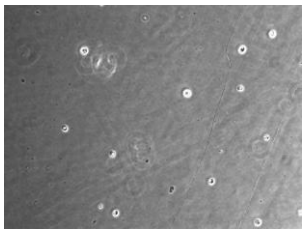
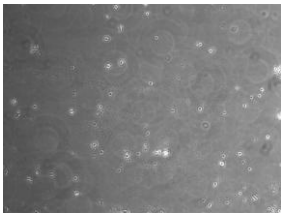
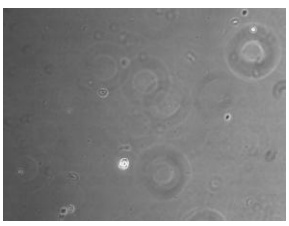
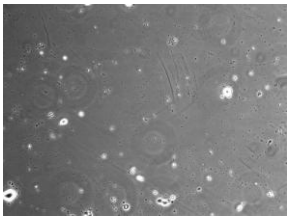


Figure A.1.3(B): *The DNA histogram for sample A03 with G1_CV =10.46.*

Photos			
 ***Figure A.1.3(C):*** *T-47D with Tergitol + SDS soaps, Solution A1- 10 minutes.*	 ***Figure A.1.3(D):*** *T-47D with Triton-x soap, Solution A4- 10 minutes.*	 ***Figure A.1.3(E):*** *T-47D with Triton-x + SDS soaps, Solution A4 + A5 - 10 minutes.*	 ***Figure A.1.3(F):*** *T98G with Tergitol soap, Solution A1 - 10 minutes.*
 ***Figure A.1.3(G):*** *T98G with Tergitol +SDS soaps, Solution*	 ***Figure A.1.3(H):*** *T98G with Triton-x +SDS soaps, Solution*	 ***Figure A.1.3(I):*** *T98G with Triton-x soap, Solution A4 - 10*	

A1 + A5 - 10 minutes.	*A4 + A5 - 10 minutes.*	*minutes.*	

EXPERIMENT 5		
Date	23.03.2011	
Used cell types and solutions with staining time		
Cell Type	T-47D	T98G
Stock Solution	Stock Solution1	
Solution A	Solution A5, 10 , 15 and 20 minutes	
Solution B	Solution B, 10 minutes	
Solution C	Solution C, minimum 20 minutes	
Citrate Buffer	Citrate Buffer, **20** minutes	
NaCl	0.9% NaCl	
The Results of the Experiment		
FCM	The results were not good enough to be considered. Therefore only one of the samples will be presented here:	

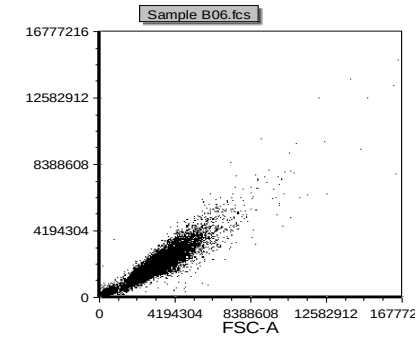


Figure A.1.4(A): *The dot plot for sample A03, T-47D with solution A5, 20 minutes.*

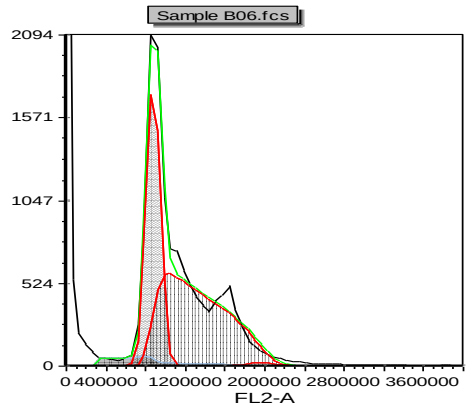


Figure A.1.3(B): *The DNA histogram for sample A03 with G1_CV =7.44.*

Photos		

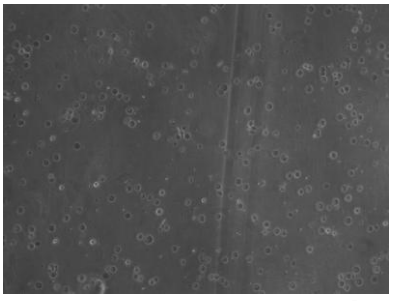
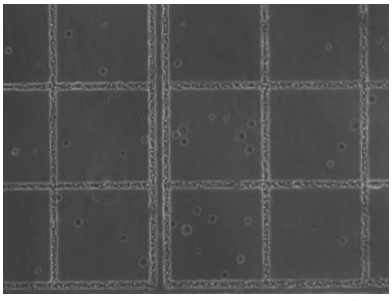
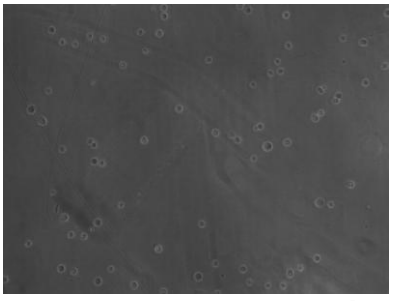
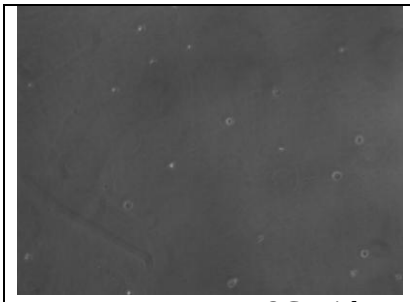
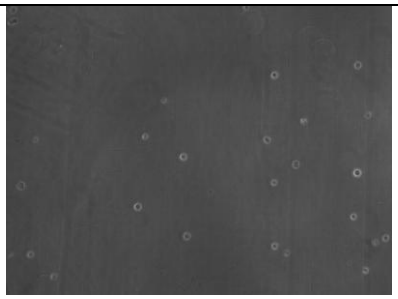
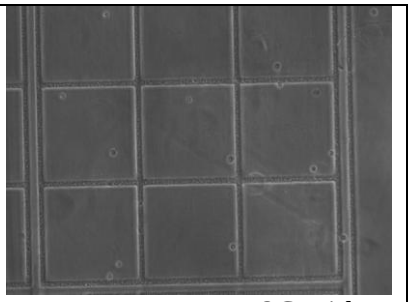

		

Figure A.1.4(C): *T-47D with solution A5, 10 minutes.*	**Figure A.1.4(D):** *T-47D with solution A5, 15 minutes.*	**Figure A.1.4(E):** *T-47D with solution A5, 20 minutes.*

 **Figure A.1.4(F):** *T98G with solution A5, 10 minutes.*	 **Figure A.1.4(G):** *T98G with solution A5, 15 minutes.*	 **Figure A.1.4(H):** *T98G with solution A5, 20 minutes.*

EXPERIMENT 6		
Date	25.03.2011	EVENT(Cell) number : 20 000
Used cell types and solutions with staining time		
Cell Type	T-47D	T98G
Stock Solution	Stock Solution1	
Solution A	Solution A5, 20 and 35 minutes	
Solution B	Solution B, 10 minutes	
Solution C	Solution C, minimum 20 minutes	
Citrate Buffer	Citrate Buffer, **30** minutes	
NaCl	0.9% NaCl	
The Results of the Experiment		
FCM	The results were not good enough to be considered. Therefore only one of the samples will be presented here:	

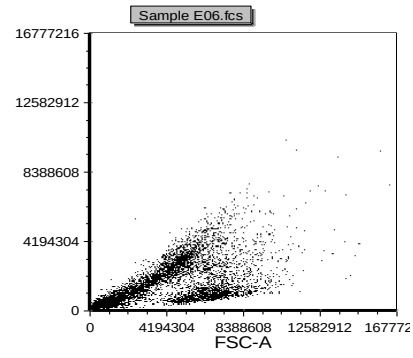


Figure A.1.5(A): *The dot plot for sample E06, T98G with solution A5, 35 minutes.*

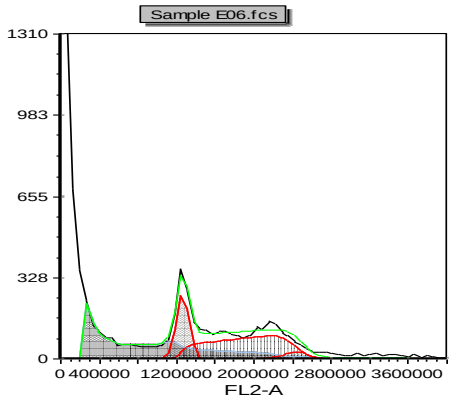


Figure A.1.5(B): *The DNA histogram for sample E06 with G1_CV =4.97.*

Photos	

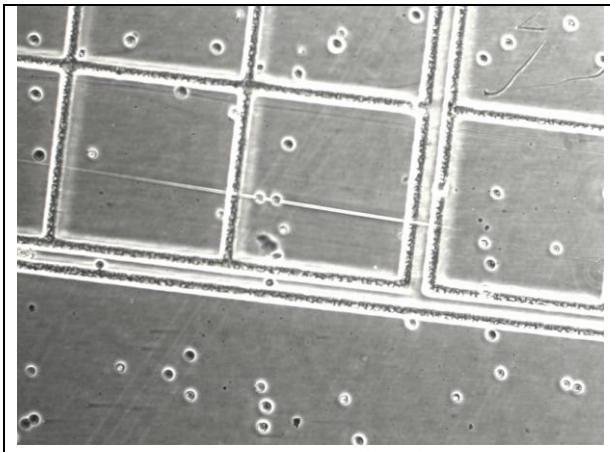


Figure A.1.5(C): *T-47D with solution A5, 20 mins.*

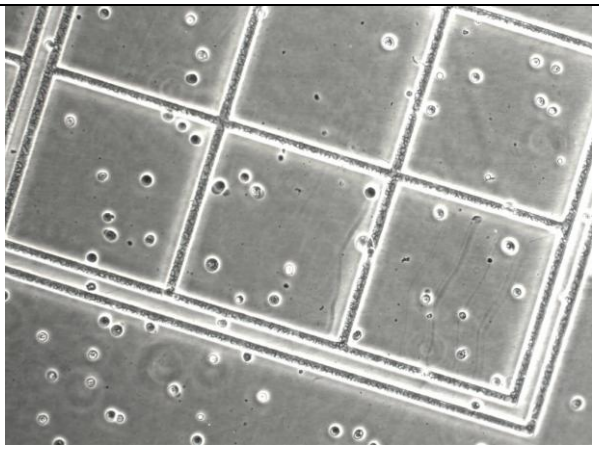


Figure A.1.5(D): *T-47D with solution A5, 35 mins.*

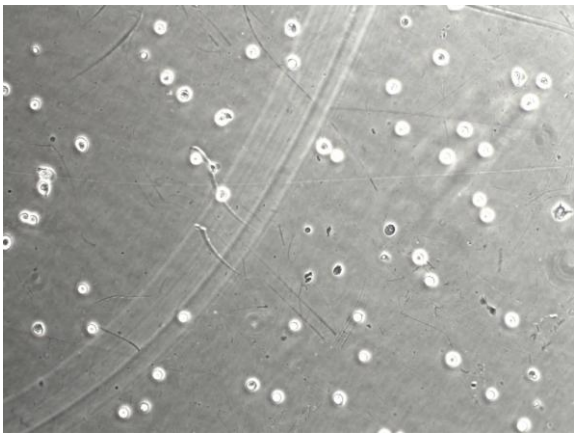


Figure A.1.5(E): *T98G with solution A5, 20 mins.*

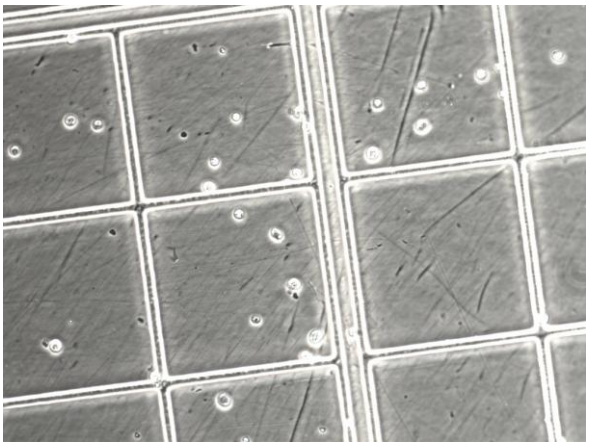


Figure A.1.5(F): *T98G with solution A5, 35 mins.*

EXPERIMENT 7			
Date	30.03.2011	EVENT(Cell) number : 20 000	
Used cell types and solutions with staining time			
Cell Type	T-47D	T98G-P	T-47D-P4
Stock Solution	Stock Solution1		
Solution A	Solution A5, 20 minutes staining at 37 ^{0}C in solution A5		
Solution B	Solution B, 10 minutes		
Solution C	Solution C, minimum 20 minutes		
Citrate Buffer	Citrate Buffer, **30** minutes		
NaCl	0.9% NaCl		
The Results of the Experiment			
FCM	Two samples will be presented:		

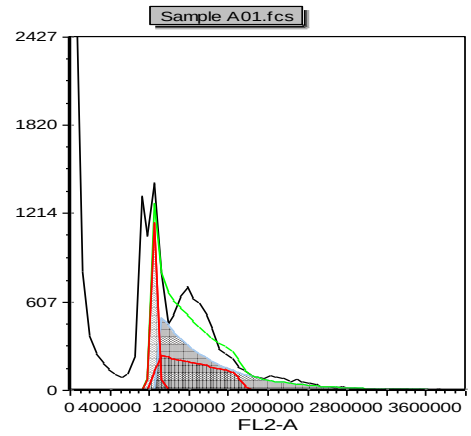


Figure A.1.6(A): *The DNA histogram for sample A01, T-47D-P4 with solution A5, 20 minutes staining at 37 ^{0}C with G1_CV =3.10.*

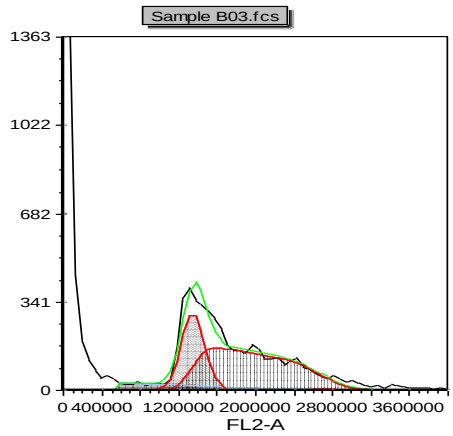


Figure A.1.6(B): *The DNA histogram for sample B03, T98G with solution A5, 20 minutes staining at 37 ^{0}C with G1_CV =8.60.*

Photos

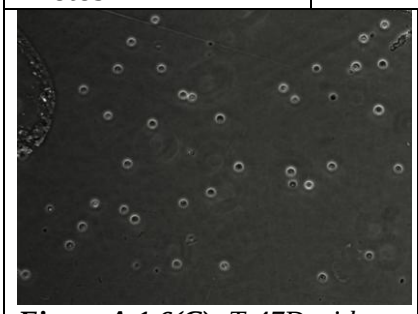


Figure A.1.6(C): *T-47D with solution A5, 20 minutes staining at 37 ^{0}C in solution A5.*

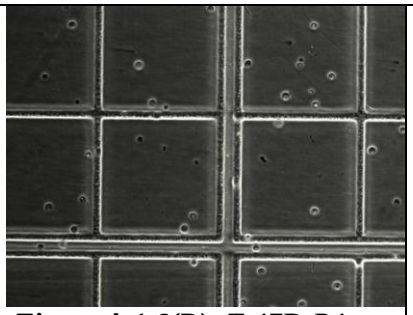


Figure A.1.6(D): *T-47D-P4 with solution A5, 20 minutes staining at 37 ^{0}C in solution A5.*

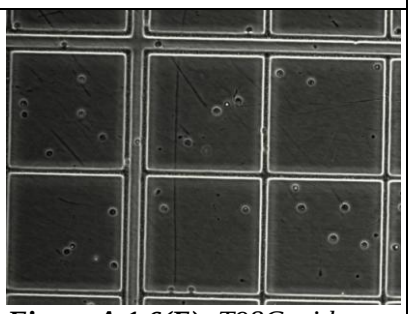


Figure A.1.6(E): *T98G with solution A5, 20 minutes staining at 37 ^{0}C in solution A5.*

EXPERIMENT 8			
Date	31.03.2011	EVENT(Cell) number : 20 000	
Used cell types and solutions with staining time			
Cell Type	T-47D	T98G-P	T-47D-P4
Stock Solution	Stock Solution1		
Solution A	Solution A5, 20 minutes staining at 37 ^{0}C in solution A5		
Solution B	Solution B, 10 minutes		
Solution C	Solution C, minimum 20 minutes		
Citrate Buffer	Citrate Buffer, **30** minutes		
NaCl	0.9% NaCl		
The Results of the Experiment			
FCM	No FCM		
Photos	Only it was seen by eyes in the microscope. Due to bad results, no photos were taken.		

EXPERIMENT 9		
Date	01.04.2011	EVENT(Cell) number : 20 000
Used cell types and solutions with staining time		
Cell Type	T98G-P	
Stock Solution	Stock Solution1	
Solution A	Solution A5, 22 minutes staining at 37 ^{0}C in solution A5 Solution A5, 42 minutes staining at 20 ^{0}C in solution A5	
Solution B	Solution B, 10 minutes	
Solution C	Solution C, minimum 20 minutes	
Citrate Buffer	Citrate Buffer, **30** minutes	
NaCl	0.9% NaCl	
The Results of the Experiment		
FCM	Total four samples: Two of them are presented, the others with only G1_CVs.	

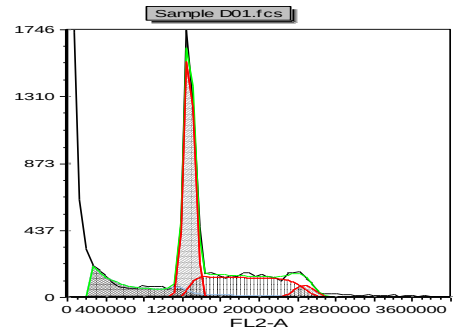


Figure A.1.7(A): *T98G-p 22 minutes solution A time with 37 ^{0}C. G1_CV is 4.05.*

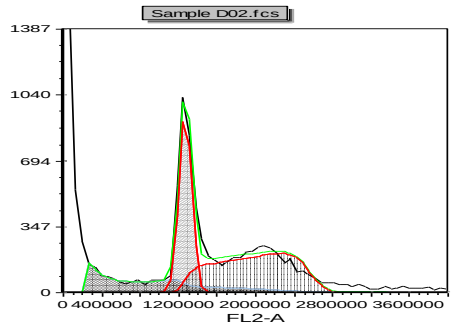


Figure A.1.7(B): *T98G-p 22 minutes solution A time with 20 ^{0}C. G1_CV is 5.05.*

G1_CVs for 22 minutes staining in Solution A5 with *37 ^{0}C = 4.05, 3.55*	G1_CVs for 42 minutes staining in Solution A5 with *20 ^{0}C = 5.05, 3.96*

Photos

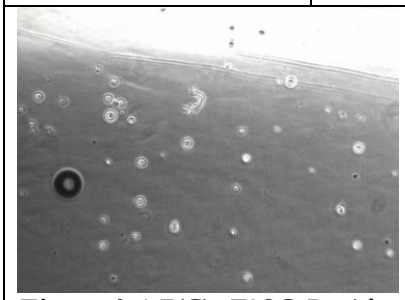


Figure A.1.7(C): *T98G-P with solution A5, 42 minutes staining at 20 ^{0}C in solution A5.*

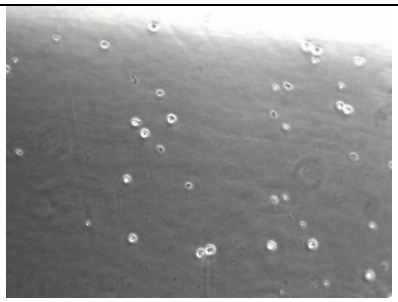


Figure A.1.7(D): *T98G-P with solution A5, 42 minutes staining at 20 ^{0}C in solution A5.*

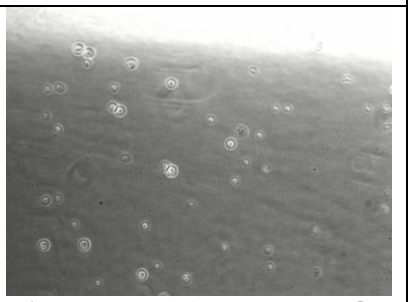


Figure A.1.7(E): *T98G-P with solution A5, 42 minutes staining at 20 ^{0}C in solution A5.*

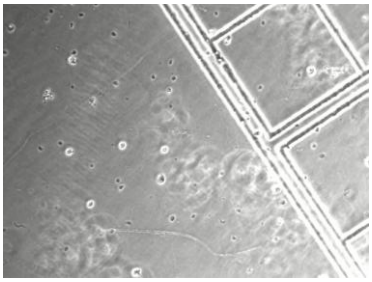


Figure A.1.7(F): *T98G-P with solution A5, 22 minutes staining*

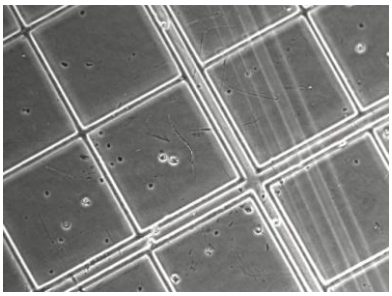


Figure A.1.7(G): *T98G-P with solution A5, 22 minutes staining*

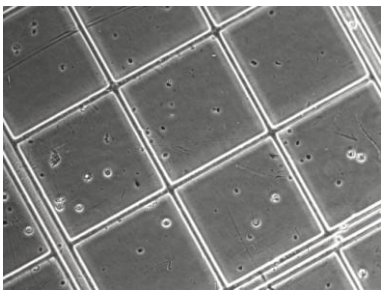


Figure A.1.7(H): *T98G-P with solution A5, 22 minutes staining*

at 37 ^{0}C in solution A5.	*at 37 ^{0}C in solution A5.*	*at 37 ^{0}C in solution A5.*

EXPERIMENT 10		
Date	06.04.2011	EVENT(Cell) number : 20 000
Used cell types and solutions with staining time		
Cell Type	T98G-P	T-47D
Stock Solution	Stock Solution1	
Solution A	Solution A5, 22 minutes staining at 37 ^{0}C in solution A5 Solution A5, 25 minutes staining at 37 ^{0}C in solution A5	
Solution B	Solution B, 10 minutes	
Solution C	Solution C, minimum 20 minutes	
Citrate Buffer	Citrate Buffer, **30** minutes	
NaCl	0.9% NaCl	
The Results of the Experiment		
FCM	Only two samples of T98G-p cells are presented. The rest has been ignored due to bad results.	

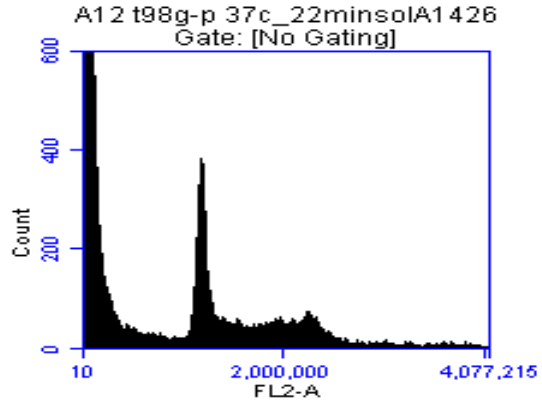


Figure A.1.8(A): *T98G-p 22 minutes solution A time with 37 ^{0}C for sample A12 with G1_CV is 4.47.*

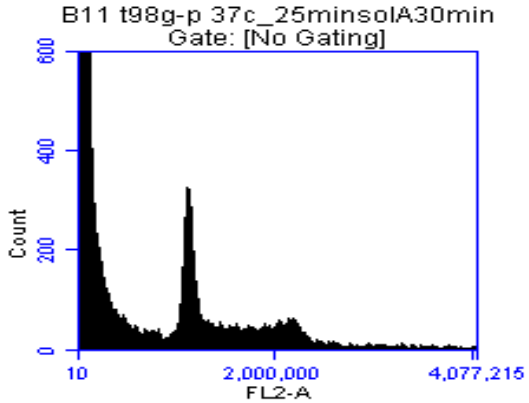


Figure A.1.8(A): *T98G-p 25 minutes solution A time with 37 ^{0}C for sample B11 with G1_CV is 3.69.*

G1_CVs for 22 minutes staining in Solution A5 with *37 ^{0}C = 3.84, 3.97, 4.00, 3.47 where average G1_CV=3.82*	G1_CVs for 25 minutes staining in Solution A5 with *37 ^{0}C = 7.55, 4.08, 3.46, 3.96 where average G1_CV =4.69.*
Photos	

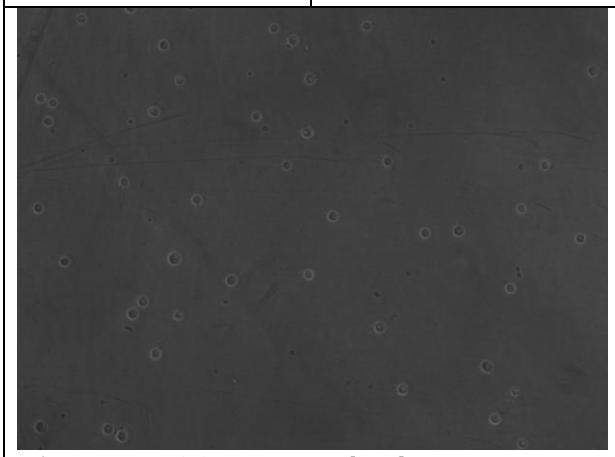


Figure A.1.8(C): *T-47D with solution A5, 22 minutes staining at 37 ^{0}C.*

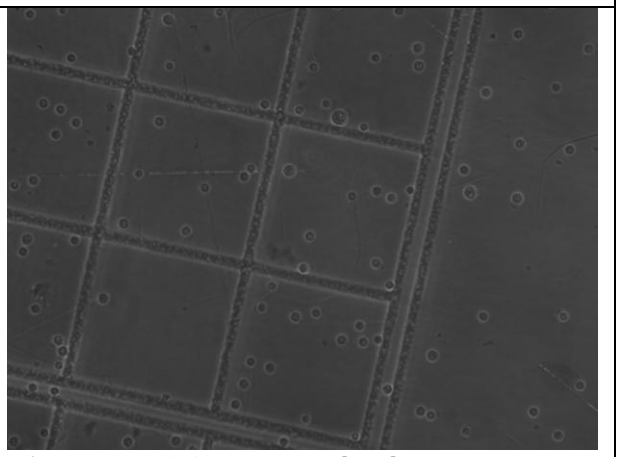


Figure A.1.8(D): *T-47D with solution A5, 25 minutes staining at 37 ^{0}C.*

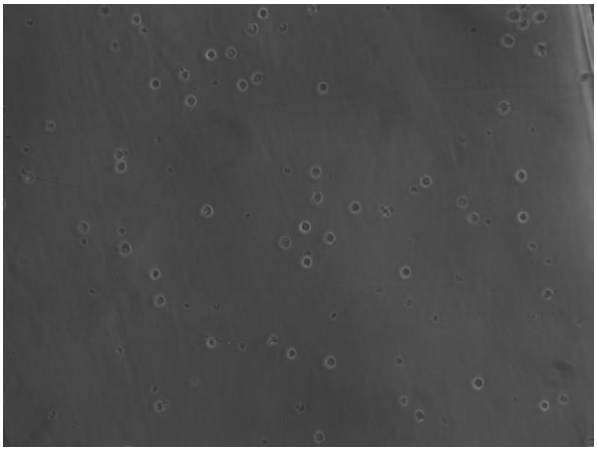


Figure A.1.8(E): *T98G-P with solution A5, 22 minutes staining at 37 ^{0}.*

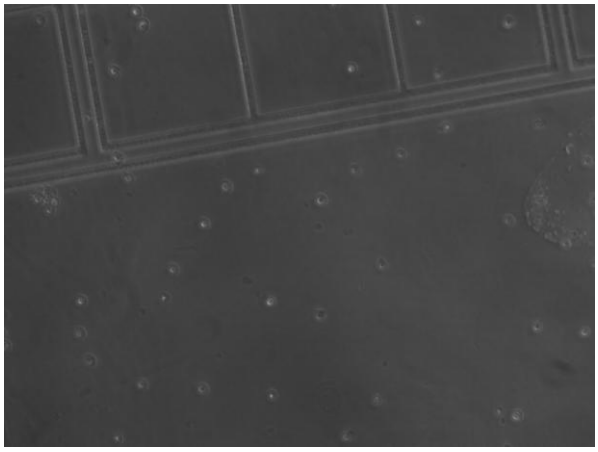


Figure A.1.8(F): *T98G-P with solution A5, 25 minutes staining at 37 ^{0}C.*

EXPERIMENT 11		
Date	13.04.2011	EVENT(Cell) number :
Used cell types and solutions with staining time		
Cell Type	T-47D	
Stock Solution	Stock Solution1	
Solution A	Solution A6, 22 minutes staining at 20 ^{0}C and 37 ^{0}C. Solution A7, 22 minutes staining at 20 ^{0}C and 37 ^{0}C.	
Solution B	Solution B, 10 minutes	
Solution C	Solution C, minimum 20 minutes	
Citrate Buffer	Citrate Buffer, 30 minutes	
NaCl	0.9% NaCl	
The Results of the Experiment		
FCM	No FCM	
Photos		

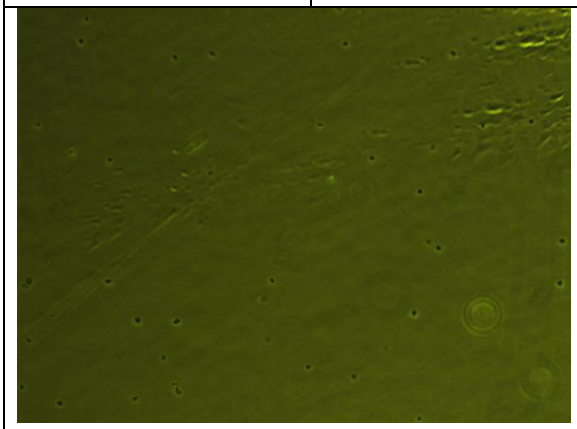


Figure A.1.9(A): *T-47D with solution A6,0.5 ‰ Tritonx-114 soap, 22 minutes staining at 20 ^{0}C.*

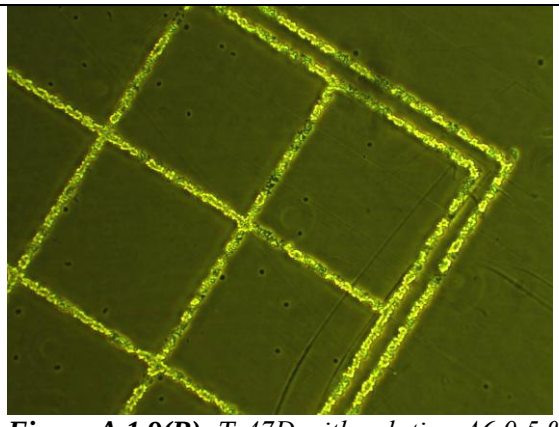


Figure A.1.9(B): *T-47D with solution A6,0.5 ‰ Tritonx-114 soap, 22 minutes staining at 37 ^{0}C.*

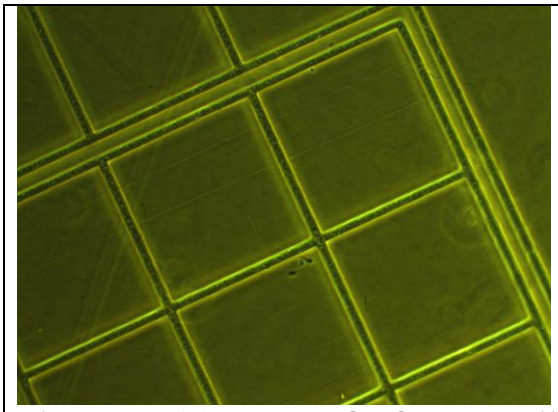


Figure A.1.9(C): *T-47D with solution A7,1 ‰ Tritonx-114 soap, 22 minutes staining at 20 ^{0}C.*

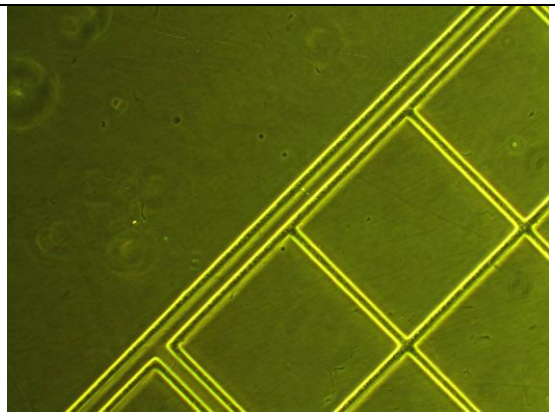


Figure A.1.9(D): *T-47D with solution A7,0.5 ‰ Tritonx-114 soap, 22 minutes staining at 37 ^{0}C.*

EXPERIMENT 12		
Date	20.04.2011	EVENT(Cell) number : 6500 -20 000
Used cell types and solutions with staining time		
Cell Type	T98G	T-47D
Stock Solution	Stock Solution1	
Solution A	Solution A6, 22 minutes staining at 20 ^{0}C and 37 ^{0}C. Solution A1, 22 minutes staining at 37 ^{0}C.	
Solution B	Solution B, 10 minutes	
Solution C	Solution C, minimum 20 minutes	
Citrate Buffer	Citrate Buffer, **30** minutes	
NaCl	0.9% NaCl	
The Results of the Experiment		
FCM		

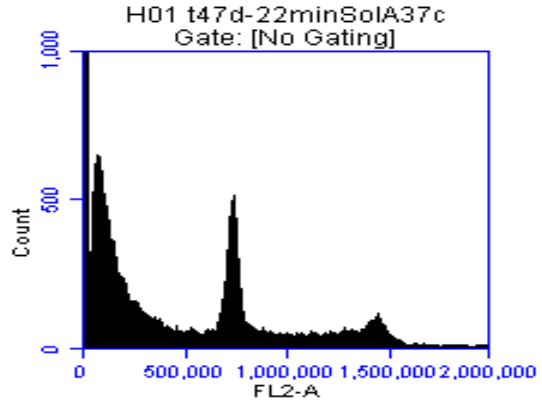


Figure A.1.10(A): *T-47D ,22 minutes solution A6 time with 37 ^{0}C for sample H01 with G1_CV is 4.21 where average G1_CV =4.07.*

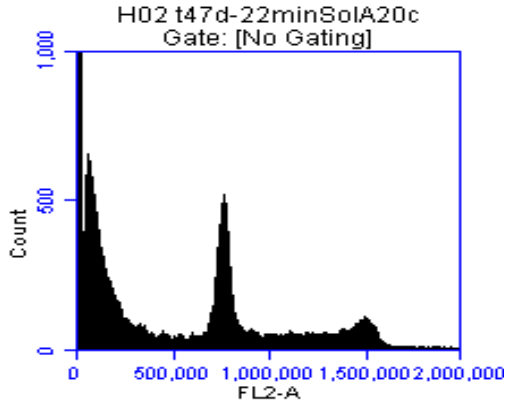


Figure A.1.10(B): *T-47D ,22 minutes solution A6 time with 20 ^{0}C for sample H21 with G1_CV is 4.31 where average G1_CV =4.25.*

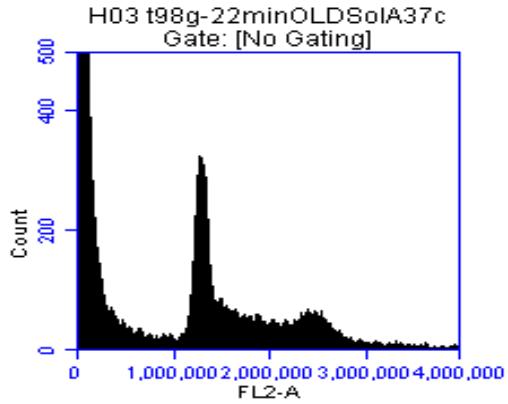


Figure A.1.10(C): *T98G, 22 minutes solution A1 time with 37 ^{0}C for sample H03 with G1_CV is 4.53 where average G1_CV =4.57.*

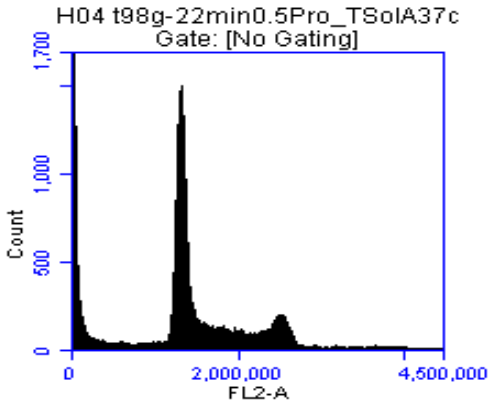


Figure A.1.10(D): *T98G, 22 minutes solution A6 time with 37 ^{0}C for sample H04 with G1_CV is 4.03 where average G1_CV =416.*

Photos

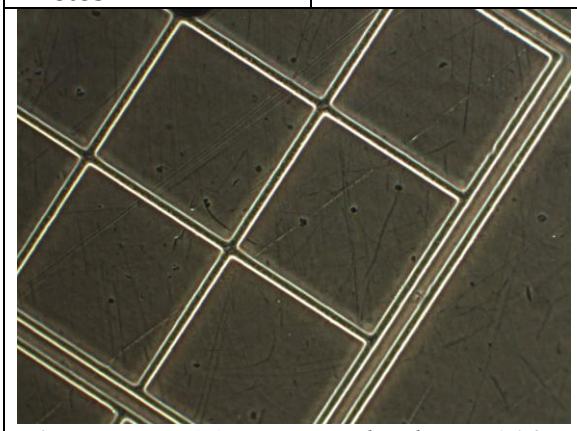


Figure A.1.10(E): *T-47D with solution A6,0.5 ‰ Tritonx-114 soap, 22 minutes staining at 20 ^{0}C.*

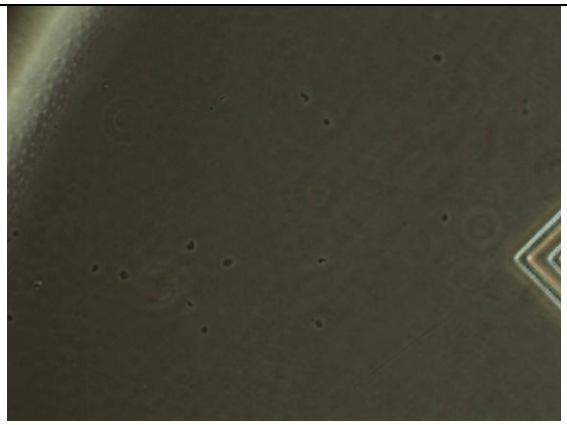


Figure A.1.10(F): *T-47D with solution A6,0.5 ‰ Tritonx-114 soap, 22 minutes staining at 37 ^{0}C.*

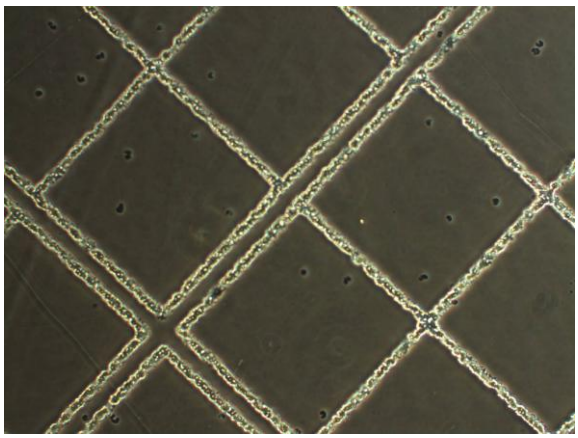


Figure A.1.10(G): *T98G with solution A6,0.5 ‰ Tritonx-114 soap, 22 minutes staining at 37 ^{0}C.*

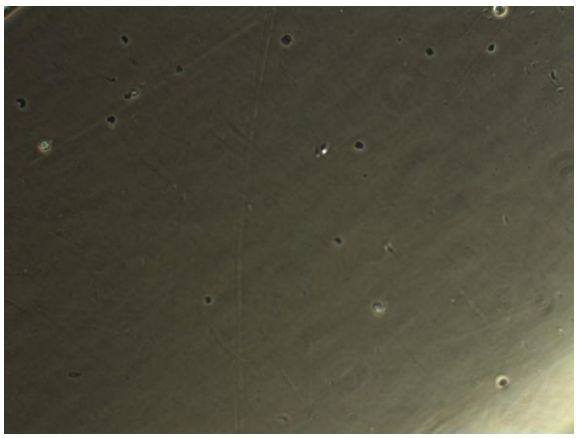


Figure A.1.10(H): *T98G with solution A1, 22 minutes staining at 37 ^{0}C.*

EXPERIMENT 13		
Date	03.05.2011	EVENT(Cell) number : 20 000
Used cell types and solutions with staining time		
Cell Type	T98G	T-47D
Stock Solution	Stock Solution1	
Solution A	Solution A6, 22 minutes staining at 20 ^{0}C Solution A6, 15 minutes staining at 37 ^{0}C. Solution A6 (less concentrated : 0.25 ‰ and 0.375 ‰ and normal 0.5 ‰)	
Solution B	Solution B, 10 minutes	
Solution C	Solution C, minimum 20 minutes	
Citrate Buffer	Citrate Buffer, **30** minutes	
NaCl	0.9% NaCl	
The Results of the Experiment		
FCM		

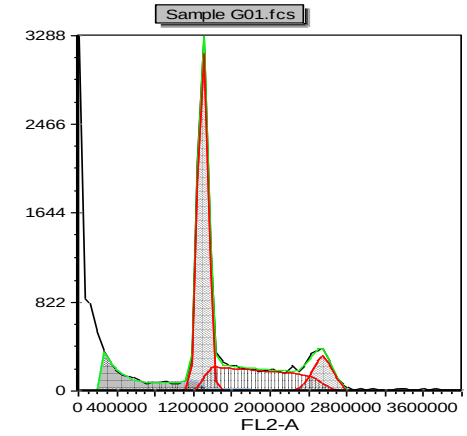


Figure A.1.11(A): *T98G with solution A6, 0.375 ‰ Tritonx-114 soap, 15 minutes staining at 20 ^{0}C with G1_CV= 3.92.*

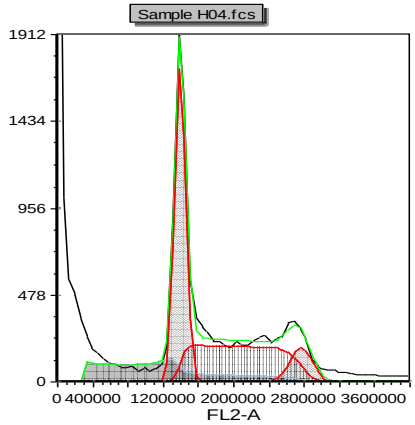


Figure A.1.11(B): *T98G with solution A6, 0.25 ‰ Tritonx-114 soap, 22 minutes staining at 20 ^{0}C with G1_CV= 4.32.*

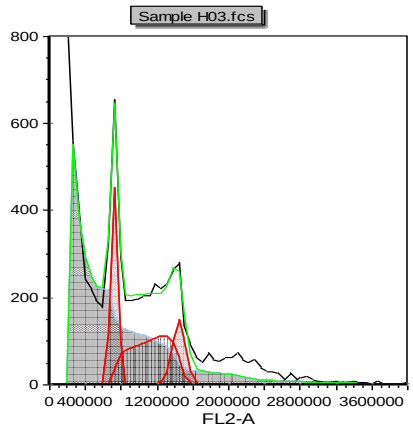


Figure A.1.11(C): *T-47D with solution A6, 0.25 ‰ Tritonx-114 soap, 22 minutes staining at 20 ^{0}C with G1_CV= 4.94.*

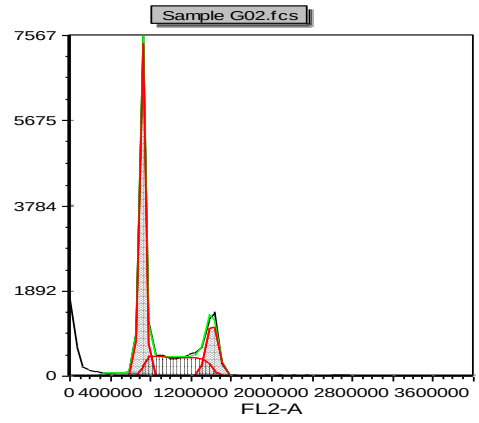


Figure A.1.11(D): *T-47D with solution A6, 0.5 ‰ Tritonx-114 soap, 15 minutes staining at 20 ^{0}C with G1_CV= 3.93.*

Photos	

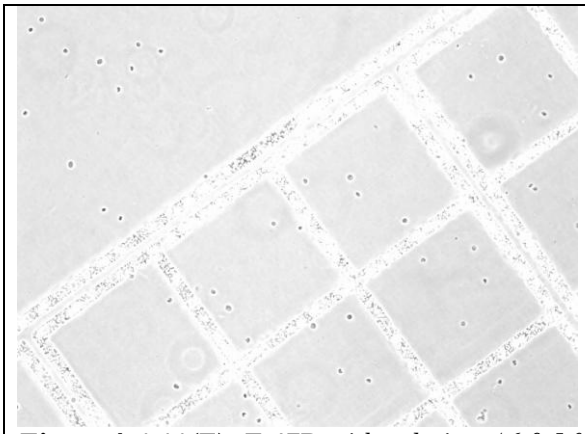


Figure A.1.11(E): *T-47D with solution A6,0.5 ‰ Tritonx-114 soap, 15 minutes staining at 20 ^{0}C.*

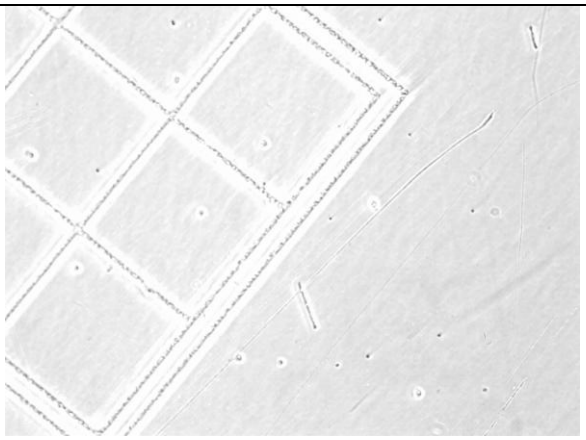


Figure A.1.11(F): *T-47D with solution A6,0.25 ‰ Tritonx-114 soap, 22 minutes staining at 20 ^{0}C.*

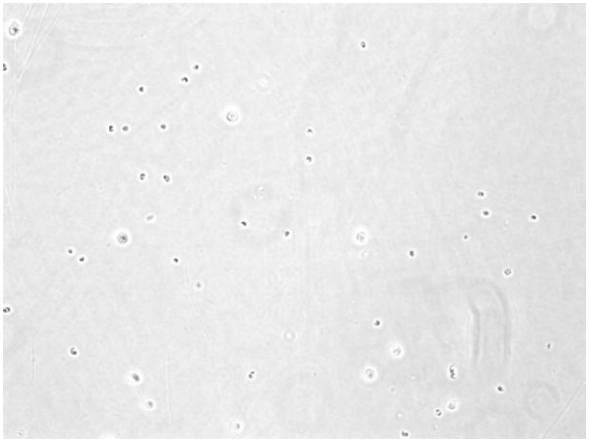


Figure A.1.11(G): *T98G with solution A8,0.375 ‰ Tritonx-114 soap, 15 minutes staining at 20 ^{0}C.*

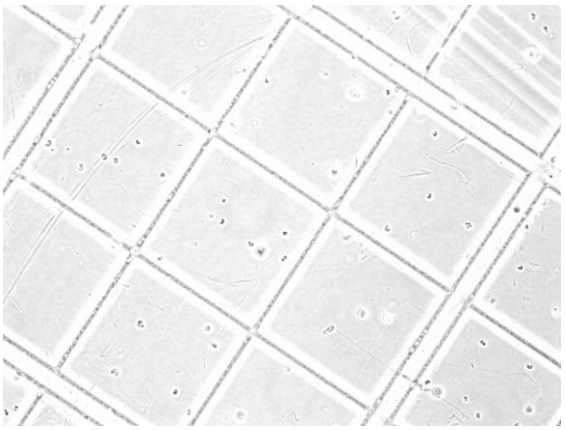


Figure A.1.11(H): *T98G with solution A6,0.25 ‰ Tritonx-114 soap, 22 minutes staining at 20 ^{0}C.*

EXPERIMENT 14		
Date	11.05.2011	EVENT(Cell) number : 20 000
Used cell types and solutions with staining time		
Cell Type	T98G	T-47D
Stock Solution	Stock Solution1	
Solution A	Solution A6 (0.4‰ Trition-x 114), 15 minutes staining at 20 ^{0}C	
Solution B	Solution B, 10 minutes	
Solution C	Solution C, minimum 20 minutes	
Citrate Buffer	Citrate Buffer, **30** minutes	
NaCl	0.9% NaCl	
The Results of the Experiment		
FCM		

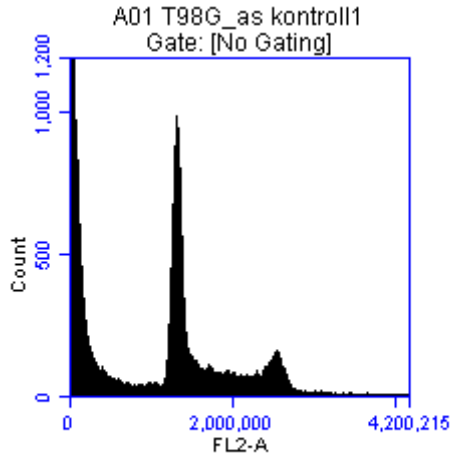


Figure A.1.12(A): *T98G as control with solution A8, 0.4 ‰ Tritonx-114 soap, 15 minutes staining at 20 ^{0}C with G1_CV= 3.92 where average G1_CV for three control samples is 4.02 ±0.10.*

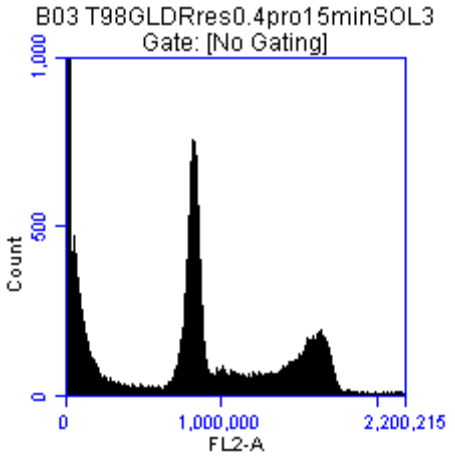


Figure A.1.12(B): *T98G LDRres with solution A8, 0.4 ‰ Tritonx-114 soap, 15 minutes staining at 20 ^{0}C with G1_CV= 4.82 where average G1_CV for three control samples is 5.12 ± 0.13.*

Table A.1.1: *Cell Cycle distribution for T98G and T98G LDRres cells.*

Experiment 14			
Cell Type, 3 samples each	G1 %	S%	G2%
T98G (Control)	49,87 ± 2,74	38,29 ± 3,31	11,83 ± 1,32
T98G LDRres	49,66 ± 2,81	30,03 ± 0,52	20,04 ± 2,33

Photos

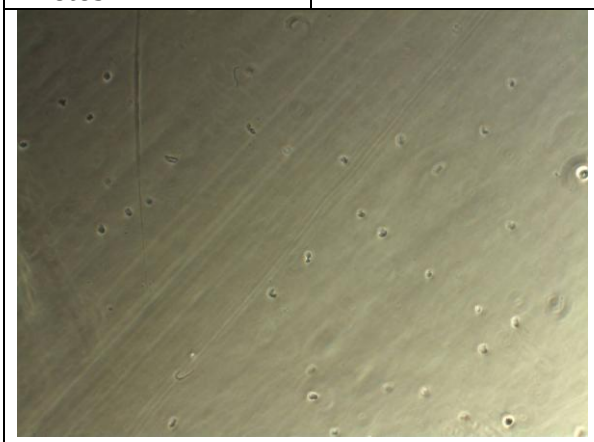


Figure A.1.12(D): *T98G as control with solution A8,0.4 ‰ Tritonx-114 soap, 15 minutes staining at 20 ^{0}C.*

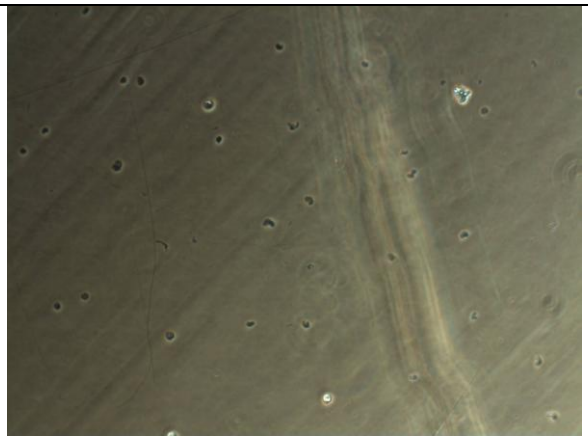


Figure A.1.12(E): *T98G as control with solution A8,0.4 ‰ Tritonx-114 soap, 15 minutes staining at 20 ^{0}C.*

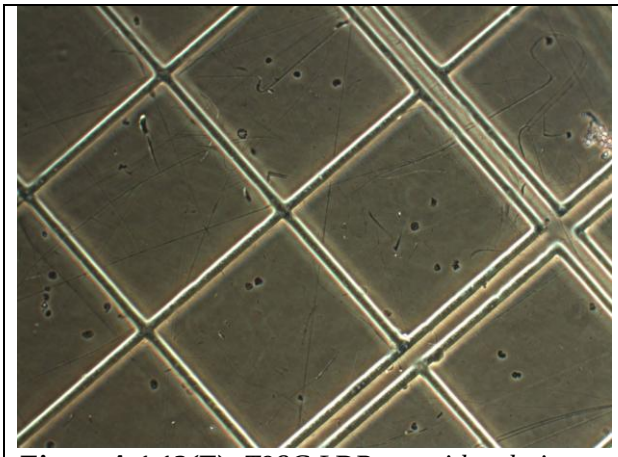


Figure A.1.12(F): *T98G LDRres with solution A8,0.4 ‰ Tritonx-114 soap, 15 minutes staining at 20 ^{0}C.*

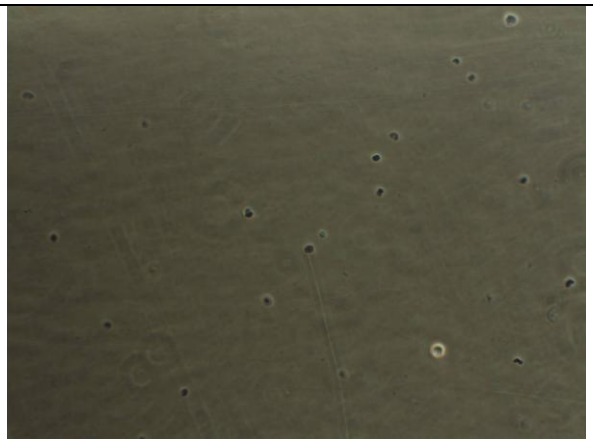


Figure A.1.12(G): *T98G LDRres with solution A8,0.4 ‰ Tritonx-114 soap, 15 minutes staining at 20 ^{0}C.*

EXPERIMENT 15		
Date	15.06.2011	EVENT(Cell) number : 20 000
Used cell types and solutions with staining time		
Cell Type	T-47D	
Stock Solution	Stock Solution1	
Solution A	Solution A6, 22 minutes staining at 20 ^{0}C	
Solution B	Solution B, 10 minutes	
Solution C	Solution C, minimum 20 minutes	
Citrate Buffer	Citrate Buffer, **30** minutes	
NaCl	0.9% NaCl	
The Results of the Experiment		
FCM		

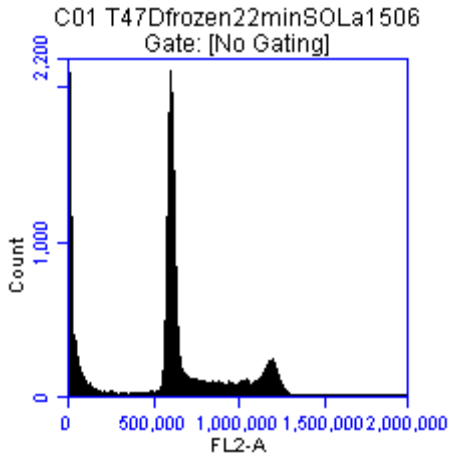


Figure A.1.13(A): *T-47D Frozen cells with solution A6, 0.5 ‰ Tritonx-114 soap, 22 minutes staining at 20 ^{0}C with G1_CV= 4.12.*

Table A.1.2: *Cell Cycle distribution for T-47D Frozen cells.*

Experiment 15			
Cell Type , 1 sample	G1 %	S%	G2%
T-47D Frozen	58,54	29,68	11,78

Photos	

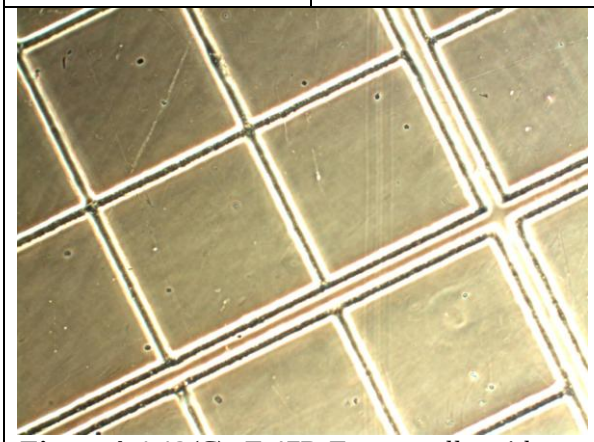


Figure A.1.13(C): *T-47D Frozen cells with solution A6, 0.5 ‰ Tritonx-114 soap, 22 minutes staining at 20 ^{0}C.*

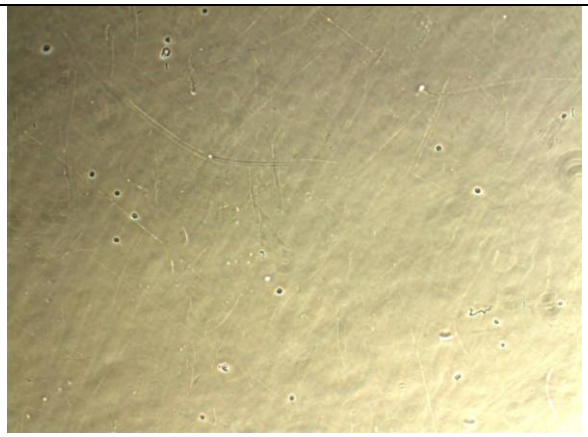


Figure A.1.13(D): *T98G as control with solution A6,0.5 ‰ Tritonx-114 soap, 22 minutes staining at 20 ^{0}C.*

EXPERIMENT 16		
Date	15.06.2011	EVENT(Cell) number : 20 000
Used cell types and solutions with staining time		
Cell Type	T98G	T98G LDRres
Stock Solution	Stock Solution1	
Solution A	Solution A8, 15 minutes staining at 20 ^{0}C	
Solution B	Solution B, 10 minutes	
Solution C	Solution C, minimum 20 minutes	
Citrate Buffer	Citrate Buffer, **30** minutes	
NaCl	0.9% NaCl	
The Results of the Experiment		
FCM		

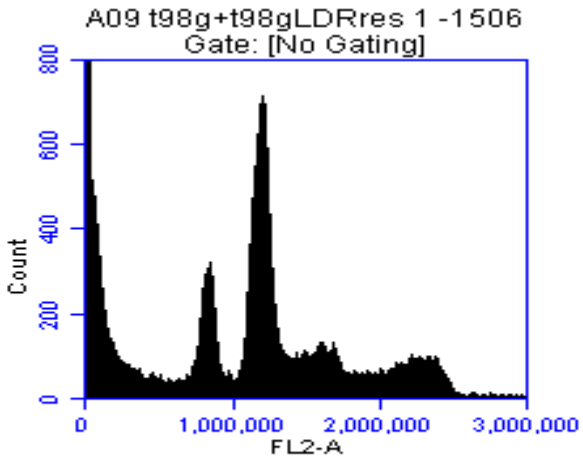


Figure A.1.14(A): *50 % T98G + 50 % T98G LDRres cells with solution A8, 0.4 ‰ Tritonx-114 soap, 15 minutes staining at 20 ^{0}C. G1_CV =4.60 for T98G LDRres with average G1_CV =4.62. G1_CV =4.60± 0.09 for T98G with average G1_CV =4.63 ± 0.09.*

Table A.1.3: *Cell Cycle distribution for 50 % T98G + 50 % T98G LDRres cells .*

Experiment 16			
Cell Type, 3 samples each	G1 %	S%	G2%
T98G (Control)	50,75 ± 1,7	36,82 ± 1,49	12,44 ± 0,31
T98G LDRres	47,78 ± 10,17	33,20 ± 14,96	19,02 ± 4,57

Photos

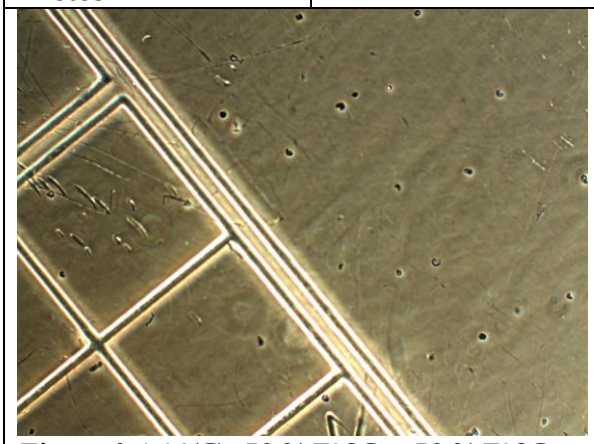


Figure A.1.14(C): *50 % T98G + 50 % T98G LDRres cells with solution A8, 0.4 ‰ Tritonx-114 soap, 15 minutes staining at 20 ^{0}C.*

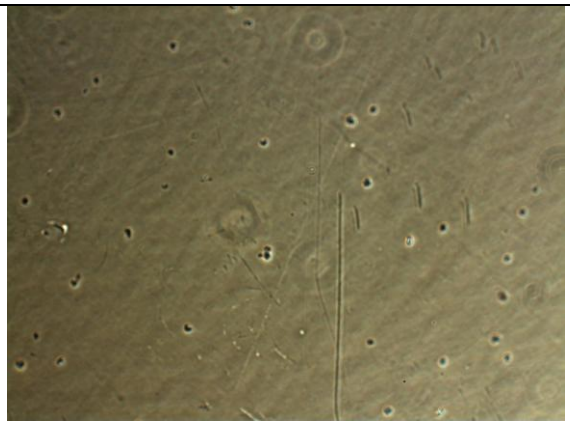
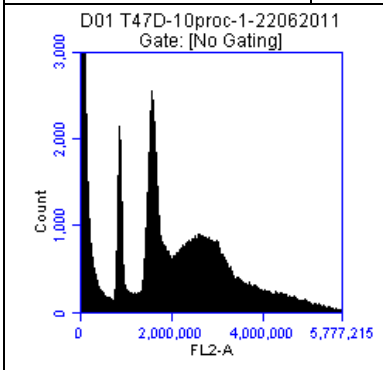
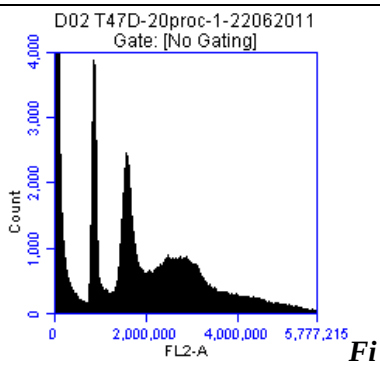


Figure A.1.14(D): *50 % T98G + 50 % T98G LDRres cells with solution A8, 0.4 ‰ Tritonx-114 soap, 15 minutes staining at 20 ^{0}C.*

EXPERIMENT 17		
Date	22.06.2011	EVENT(Cell) number : 20 000
Used cell types and solutions with staining time		
Cell Type	T47D	T98G
Stock Solution	Stock Solution1	
Solution A	Solution A6, 22 minutes staining at 20 ^{0}C Solution A8, 22 minutes staining at 20 ^{0}C	
Solution B	Solution B, 10 minutes	
Solution C	Solution C, minimum 20 minutes	
Citrate Buffer	Citrate Buffer, **30** minutes	
NaCl	0.9% NaCl	
The Results of the Experiment		
FCM		





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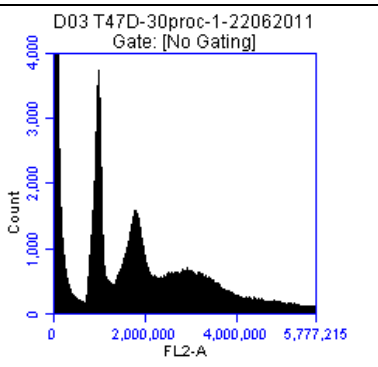


Figure A.1.15(C): *30 % T-47D*

Figure A.1.15(A): *10 % T-47D + 90 % T98G cells .*	***gure A.1.15(B):*** *20 % T-47D + 80 % T98G cells .*	*+ 70 % T98G cells .*

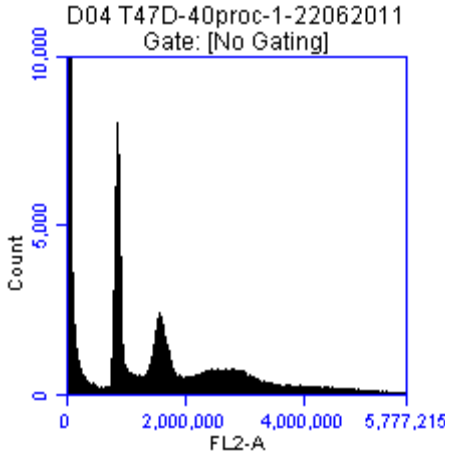


Figure A.1.15(D): *40 % T-47D + 90 % T98G cells .*

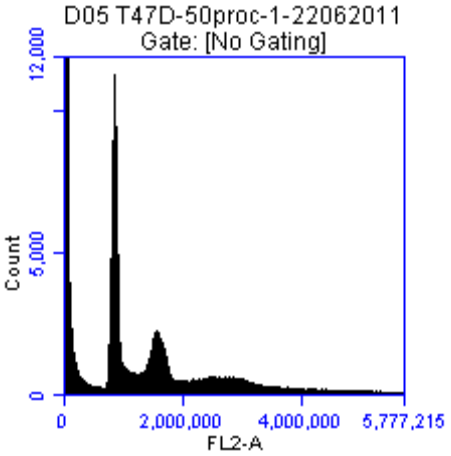


Figure A.1.15(E): *50 % T-47D + 50 % T98G cells .*

Photos	No photos

EXPERIMENT 18		
Date	29.06.2011	EVENT(Cell) number : 20 000
Used cell types and solutions with staining time		
Cell Type	T47D	T98G LDRres
Stock Solution	Stock Solution1	
Solution A	Solution A6, 22 minutes staining at 20 ^{0}C Solution A8, 22 minutes staining at 20 ^{0}C	
Solution B	Solution B, 10 minutes	
Solution C	Solution C, minimum 20 minutes	
Citrate Buffer	Citrate Buffer, **30** minutes	
NaCl	0.9% NaCl	
The Results of the Experiment		
FCM		

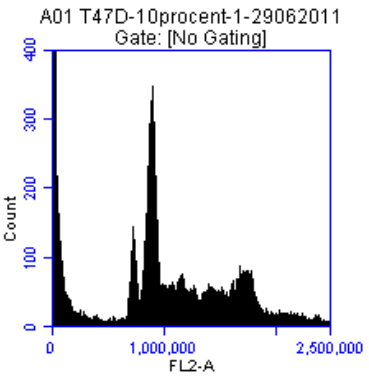
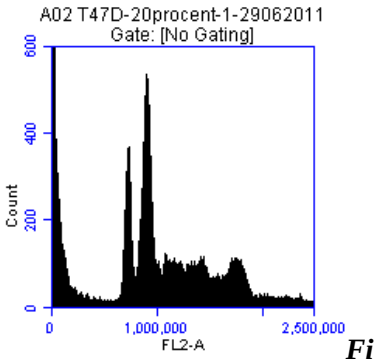


Figure A.1.16(A): *10 % T-47D*



Fi gure A.1.16(B): *20 % T-47D +*

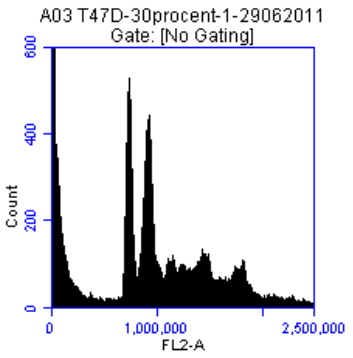


Figure A.1.16(C): *30 % T-47D + 70 % T98G LDRres cells .*

+ 90 % T98G LDRres cells .	80 % T98G LDRres cells .	

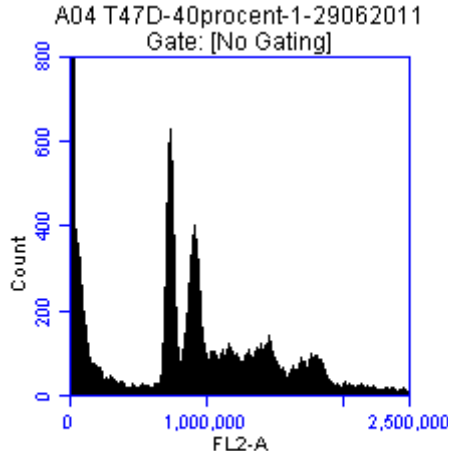


Figure A.1.16(D): *40 % T-47D + 90 % T98G LDRres cells .*

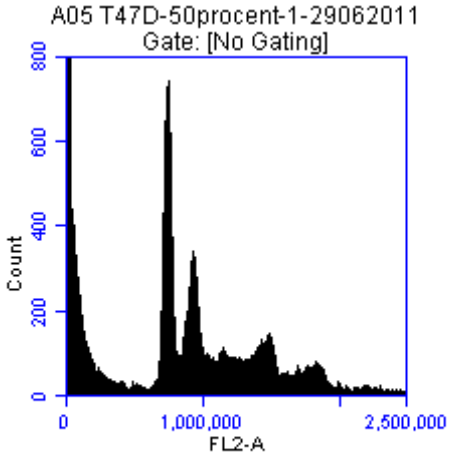


Figure A.1.16(E): *50 % T-47D + 50 % T98G LDRres cells .*

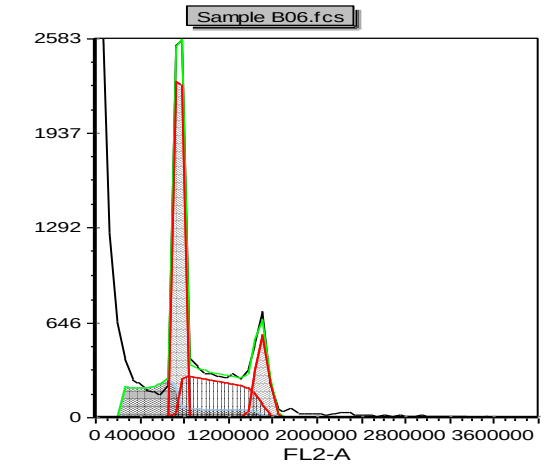


Figure A.1.16(F): *100 % T-47D with G1_CV =3.52 where average G1_CV =3.78 ± 0.197.*

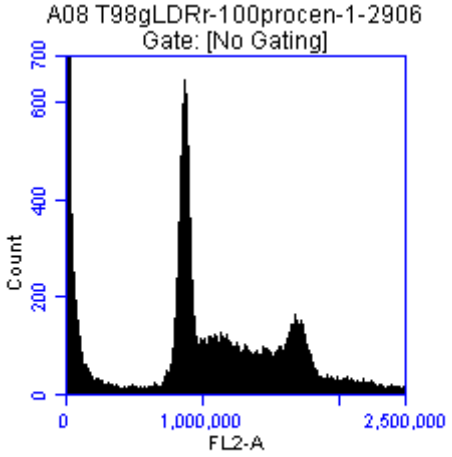


Figure A.1.16(G): *100 % T98G LDRres with G1_CV =4.67 where average G1_CV =4.65 ± 0.024. .*

Table A.1.4: *Cell Cycle distribution for both 100 % T98G LDRres and 100 % T-47D cells .*

Experiment 18			
Cell Type, 2 samples each	G1 %	S%	G2%
T-47D	55,40 ± 0,54	31.13 ± 0,45	13,46 ± 0,09
T98G LDRres	38,38 ± 0,84	53,61 ± 2,89	13,00 ± 0,69
Photos	No photos		

EXPERIMENT 19		
Date	01.07.2011	EVENT(Cell) number : 20 000
Used cell types and solutions with staining time		
Cell Type	F10 (T-47D)	F44 (T-47D)
Stock Solution	Stock Solution1	
Solution A	Solution A6, 22 minutes staining at 20 ^{0}C	
Solution B	Solution B, 10 minutes	
Solution C	Solution C, minimum 20 minutes	
Citrate Buffer	Citrate Buffer, **30** minutes	

NaCl	0.9% NaCl
The Results of the Experiment	
FCM	

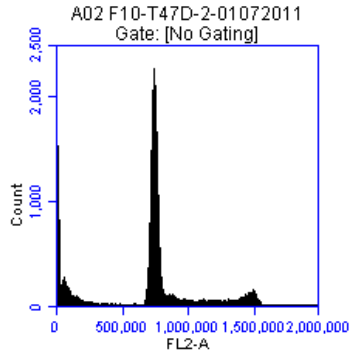


Figure A.1.17(A): *DNA histogram for 100 % F10 (T-47D) cells with G1_CV = 3.27 where average G1_CV =3.64 ± 0.21.*

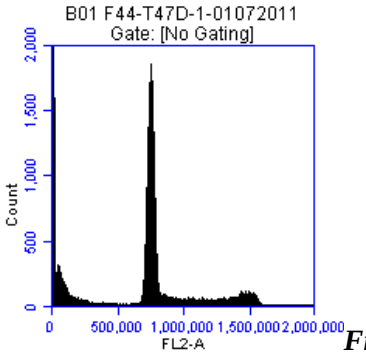


Figure A.1.17(B): *DNA histogram for 100 % F44 (T-47D) cells with G1_CV = 3.78 where average G1_CV =3.89 ± 0.13.*

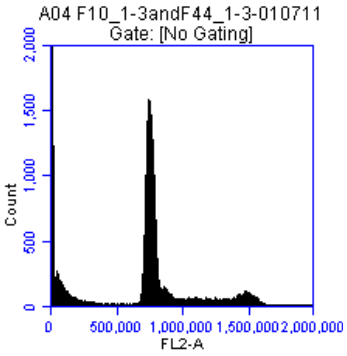


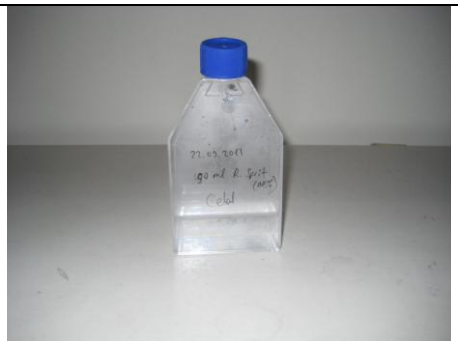
Figure A.1.17(C): *DNA histogram for 100 % F10 (F-47D) 1-3 + F44 (T-47D) 1-3 cells with G1_CV = 4.31.*

Table A.1.5: *Cell Cycle distribution for 100 % F10 (T-47D), F44 (T-47D) and for mixture of F10 1-3 + F44 1-3 cells .*

Experiment 19			
Cell Type, 3 samples each	G1 %	S%	G2%
F10 (T-47D)	70,19 ± 7,33	23,56 ± 8,99	6,24 ± 2,36
F44 (T-47D)	74,46 ± 5,59	19,49 ± 4,50	6,05 ± 1,11

Photos	No photos

Appendix A.2: The two-parametric DNA – Protein Method

The Solutions		
 ***Figure A.2.1(A):*** *PBS (1X): 900 ml MilliQ water + 100 ml PBS (10X).*	 ***Figure A.2.1(B):*** *Rectified Spirit: 900 ml MilliQ water + 100 ml PBS (10X).*	 ***Figure A.2.1(C):*** *Rectified Spirit (98 %) produced by Kemwtyl Norge AS.*

RNase Solution	RNase (60 µg/ml) in PBS (1X)
FITC Solution	FITC (0.1 µg/ml) in PBS (1X)
PI Solution	PI (34 µg/ml) in PBS (1X)

EXPERIMENT 20		
Date	19.08.2011 – 22.08.2011	EVENT(Cell) number : 20 000
Used cell types and solutions with staining time		
Cell Type	T98G	
Fixation of the cells	in 50 % PBS (1X) + 50 % Rectified Spirit in the freezer for 2 days	
First Staining	in FITC Solution and RNase Solution later left overnight at room temperature in the dark	
Second Staining	in PI Solution for minimum 1 hour	
Cleaning	with PBS (1X)	
The Results of the Experiment		
FCM		
BEFORE Compensation		AFTER Compensation

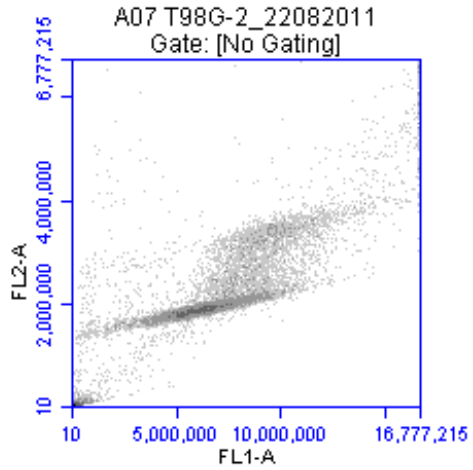


Figure A.2.2(A): *T98G, plot of DNA (FL2-A) verses Protein (FL1-A) before compensation with G1_CV =7.94.*

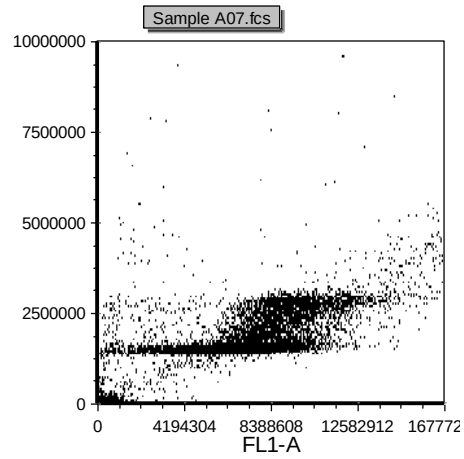


Figure A.2.2(B): *T98G, plot of DNA (FL2-A) verses Protein (FL1-A) after compensation (7.0 %) with G1_CV =4.44.*

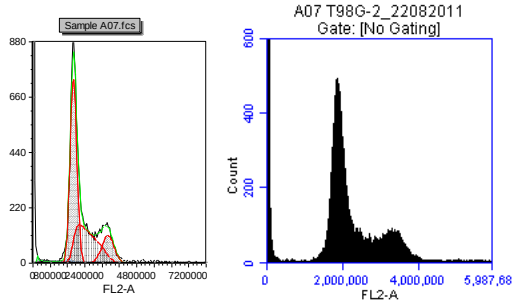


Figure A.2.2(C): *DNA (FL2-A) histograms before compensation with G1_CV =7.94.*

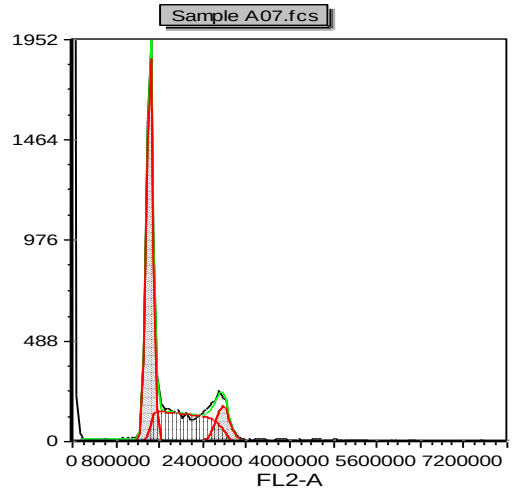


Figure A.2.2(D): *DNA (FL2-A) histogram after compensation with G1_CV =7.94.*

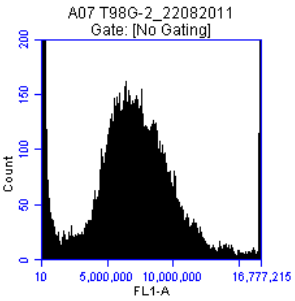


Figure A.2.2(E): *Protein (FL1-A) histogram for sample A07, no compensation.*

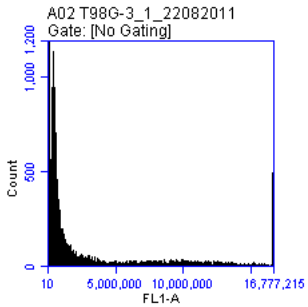


Figure A.2.2(F): *Protein (FL1-A) histogram for sample A02, no compensation.*

Table A.2.1: *Cell Cycle distribution for T98G cells before and after the compensation.*

Experiment 20			
Cell Type, 1 sample each	G1 %	S%	G2%
T98G Before Compensation	51,94	33,92	14,13
T98G After Compensation	57,65	32,23	10,13

Photos	No photos

EXPERIMENT 21		
Date	01.09.2011 – 05.09.2011	EVENT(Cell) number : 20 000
Used cell types and solutions with staining time		
Cell Type	T98G	
Fixation of the cells	in 50 % PBS (1X) + 50 % Rectified Spirit in the freezer for 4 days in 30 % PBS (1X) + 70 % Rectified Spirit in the freezer for 4 days	
First Staining	in FITC Solution and RNase Solution later left overnight at room temperature in the dark	
Second Staining	in PI Solution for minimum 1 hour	
Cleaning	with PBS (1X)	
The Results of the Experiment		
FCM		

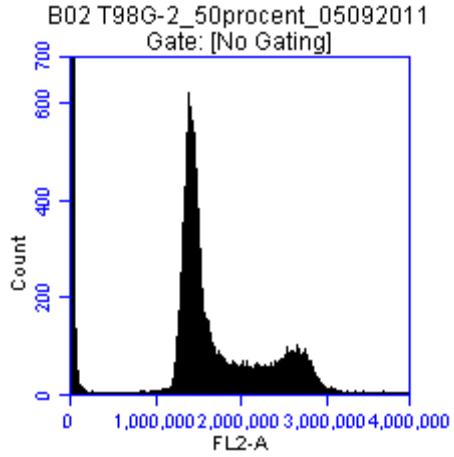


Figure A.2.3(A): *T98G, DNA (FL2-A) histogram of 50 % Rectified spirit with G1_CV =5.98 ± 0.01 where average G1_CV = 5.37 ± 0.26 .*

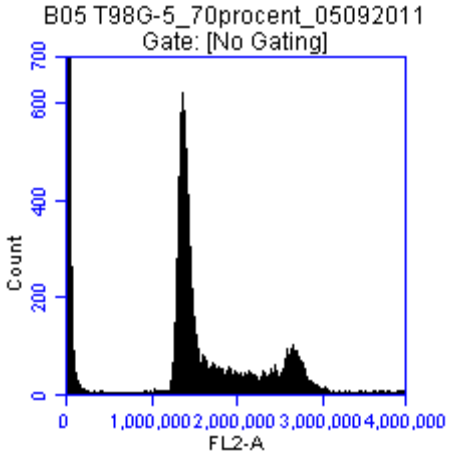


Figure A.2.3(B): *T98G, DNA (FL2-A) histogram of 70 % Rectified spirit with G1_CV =4.32 ± 0.01 where average G1_CV = 5.16 ± 0.35 .*

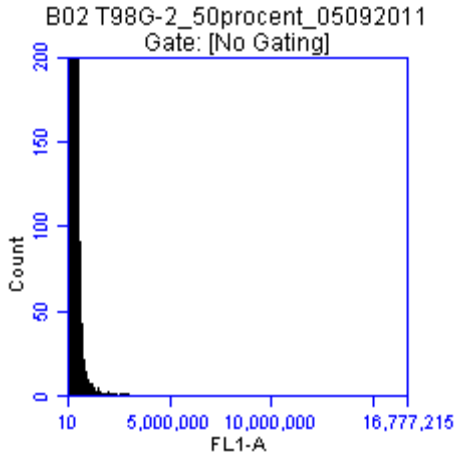


Figure A.2.3(C): *Protein (FL1-A) histogram for sample B01 with 50 % Rectified spirit.*

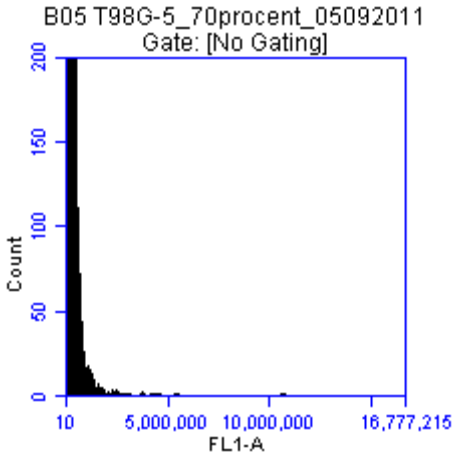


Figure A.2.2(D): *Protein (FL1-A) histogram for sample B01 with 70 % Rectified spirit.*

Table A.2.2: *Cell Cycle distribution for T98G cells both for 50 % and 70 % Rectified Spirit..*

Experiment 21			
Cell Type, 3 samples each	G1 %	S%	G2%
T98G 50 % Rectified Spirit	49,45 ± 0,65	36,55 ± 0,42	14,00 ± 0,24
T98G 70 % Rectified Spirit	47,45 ± 1,38	38,30 ± 0,88	14,24 ± 0,63

Photos	No photos

EXPERIMENT 22		
Date	07.09.2011 – 12.09.2011	EVENT(Cell) number : 20 000
Used cell types and solutions with staining time		
Cell Type	T98G	
Fixation of the cells	in 50 % PBS (1X) + 50 % Rectified Spirit in the freezer for 1 day	
First Staining	in FITC Solution and RNase Solution later left overnight at room temperature in the dark	
Second Staining	in PI Solution for minimum 1 hour	
Cleaning	with PBS (1X)	
The Results of the Experiment		
FCM		

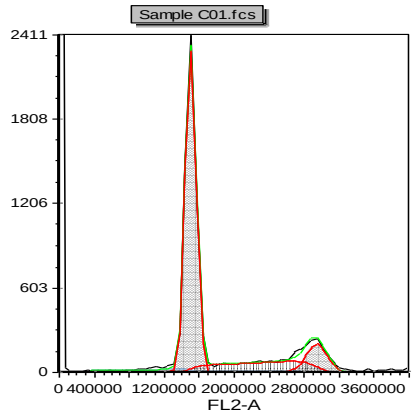


Figure A.2.4(A): *T98G, DNA (FL2-A) histogram of 50 % Rectified spirit with G1_CV =3.96 ± 0.01 where average G1_CV = 3.96 ± 0.01 .*

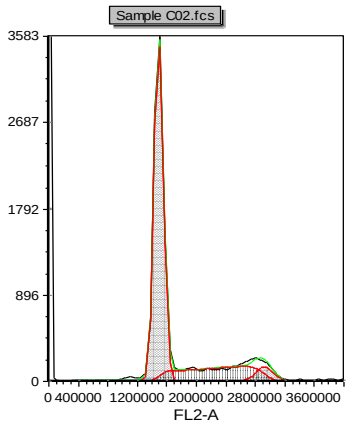


Figure A.2.3(B): *T98G, DNA (FL2-A) histogram of 50 % Rectified spirit with G1_CV =3.97 ± 0.01 where average G1_CV = 3.96 .96 ± 0.01.*

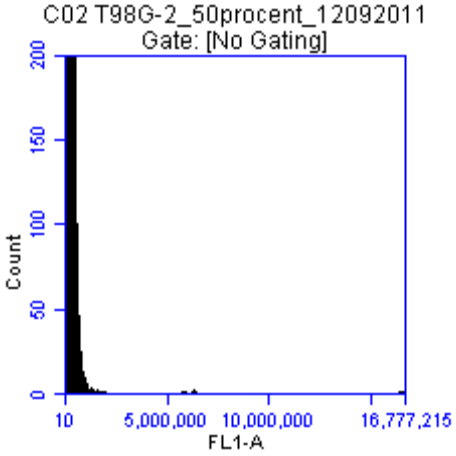


Figure A.2.4(C): *Protein (FL1-A) histogram for sample C02 with 50 % Rectified spirit.*

Table A.2.3: *Cell Cycle distribution for T98G cells both for 50 % Rectified Spirit.*

Experiment 22			
Cell Type, 2 samples	G1 %	S%	G2%
T98G 50 % Rectified Spirit	69,92 ± 0,28	21,32 ± 1,90	8,75 ± 2,18

Photos	No photos

EXPERIMENT 23		
Date	16.09.2011 – 18.09.2011	EVENT(Cell) number : 20 000
Used cell types and solutions with staining time		
Cell Type	T98G, 4 samples	
Fixation of the cells	in 50 % PBS (1X) + 50 % Rectified Spirit in the freezer for 1 day	

First Staining	in FITC Solution and RNase Solution later left overnight at room temperature in the dark
Second Staining	in PI Solution for minimum 1 hour
Cleaning	with PBS (1X)
The Results of the Experiment	
FCM	

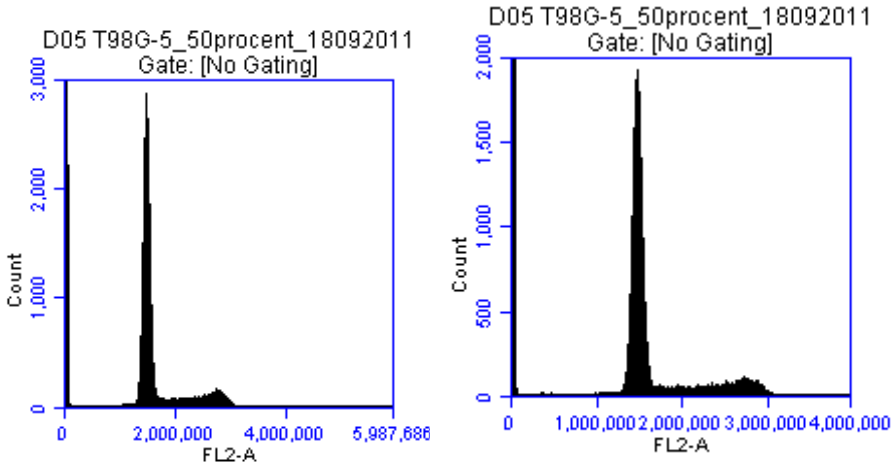


Figure A.2.5(A): *T98G, DNA (FL2-A) histogram of 50 % Rectified spirit with G1_CV =3.72 ± 0.01 where average G1_CV = 3.79 ± 0.04 .*

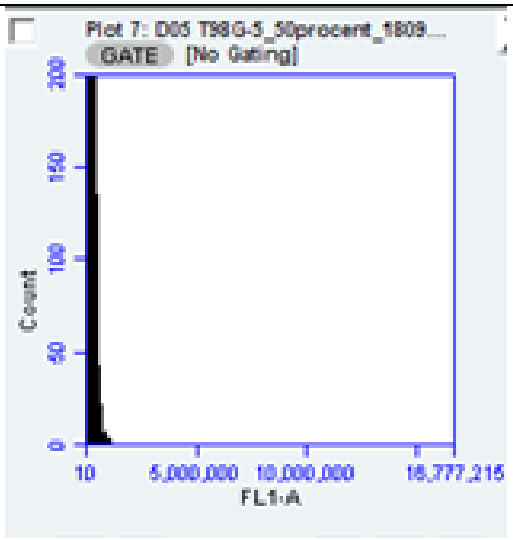


Figure A.2.5(B): *Protein (FL1-A) histogram for sample D05 with 50 % Rectified spirit.*

Table A.2.4: *Cell Cycle distribution for T98G cells both for 50 % Rectified Spirit.*

Experiment 23			
Cell Type, 4 samples	G1 %	S%	G2%
T98G 50 % Rectified Spirit	72,53 ± 0,36	23,35 ± 0,45	4,11 ± 0,18

Photos	No photos

EXPERIMENT 24		
Date	22.09.2011 – 25.09.2011	EVENT(Cell) number : 20 000
Used cell types and solutions with staining time		
Cell Type	T98G-P, 6 samples	
Fixation of the cells	in 50 % PBS (1X) + 50 % Rectified Spirit in the freezer for 1 day	
First Staining	in FITC Solution - FITC (0.1 µg/ml) in PBS (1X) in FITC Solution - FITC (1.0 µg/ml) in PBS (1X) and later left overnight at room temperature in the dark	
Second Staining	in RNase Solution for 1 hour	
Third Staining	in PI Solution for minimum 1 hour	
Cleaning	with PBS (1X)	
The Results of the Experiment		
FCM		

BEFORE Compensation

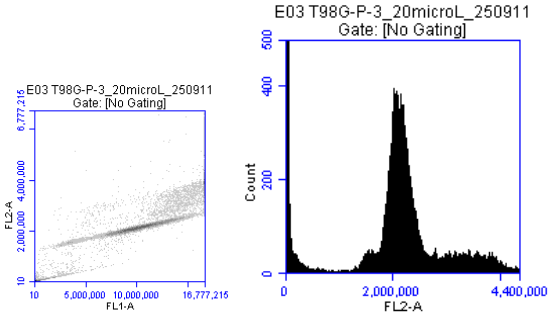


Figure A.2.6(A): *Plot of DNA (FL2-A) verses Protein (FL1-A) and the DNA histogram before compensation with G1_CV =8.63.*

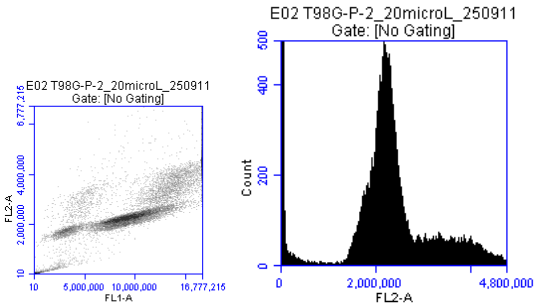


Figure A.2.6(C): *Plot of DNA (FL2-A) verses Protein (FL1-A) and the DNA histogram before compensation with G1_CV =12.01.*

AFTER Compensation

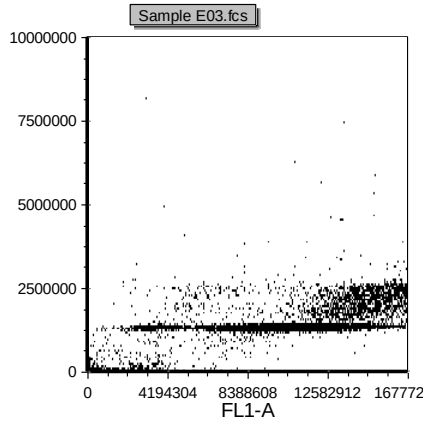


Figure A.2.6(B): *Plot of DNA (FL2-A) verses Protein (FL1-A) after compensation (8.5 %)with G1_CV =3.49.*

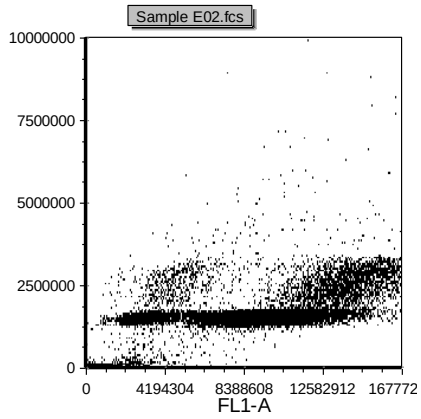


Figure A.2.6(D): *Plot of DNA (FL2-A) verses Protein (FL1-A) after compensation (8.0 %) with G1_CV =7.72.*

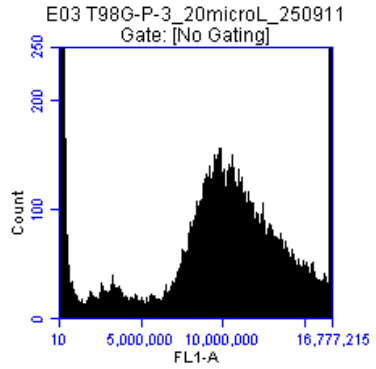


Figure A.2.6(E): *Protein (FL1-A) histogram for sample D05 with 50 % Rectified spirit and with FITC (0.1 µg/ml) in PBS (1X).*

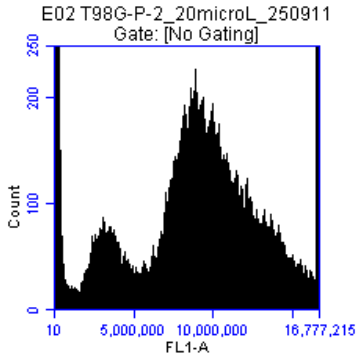


Figure A.2.6(F): *Protein (FL1-A) histogram for sample E02 with 50 % Rectified spirit and FITC (0.1µg/ml) in PBS (1X).*

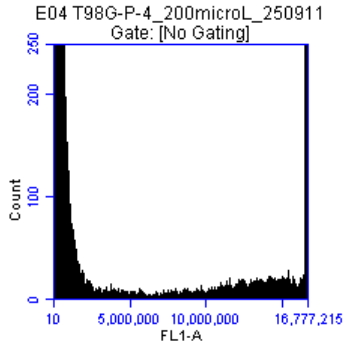


Figure A.2.6(G): *Protein (FL1-A) histogram for sample D05 with 50 % Rectified spirit and with FITC (1.0 µg/ml) in PBS (1X).*

Table A.2.5: *Cell Cycle distribution for T98G-P cells with 50 % Rectified Spirit and with FITC (0.1µg/ml) in PBS (1X).*

Experiment 24			
Cell Type, 3 samples	G1 %	S%	G2%
T98G-P 50 % Rectified Spirit - FITC (0.1 µg/ml) in PBS (1X)	72,11 ± 1,95	25,23 ± 1,69	2,65 ± 0,32

Photos	No photos

EXPERIMENT 25		
Date	30.09.2011 – 02.10.2011	EVENT(Cell) number : 20 000
Used cell types and solutions with staining time		
Cell Type	T98G	T98G-P
Fixation of the cells	in 50 % PBS (1X) + 50 % Rectified Spirit in the freezer for 1 day	
First Staining	in FITC Solution - FITC (0.1 µg/ml) in PBS (1X), left overnight at room temperature in the dark	
Second Staining	1 hour in RNase Solution	
Third Staining	Minimum 1 hour in PI Solution	
Cleaning	with PBS (1X)	
The Results of the Experiment		
FCM		
BEFORE Compensation	AFTER Compensation	

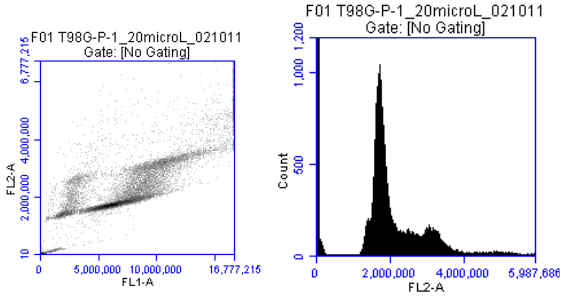


Figure A.2.7(A): *Plot of DNA (FL2-A) verses Protein (FL1-A) and the DNA histogram before compensation with G1_CV =8.72*

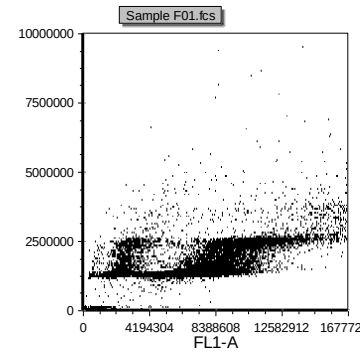


Figure A.2.7(B): *Plot of DNA (FL2-A) verses Protein (FL1-A) after compensation (7.5 %) with G1_CV =3.96.*

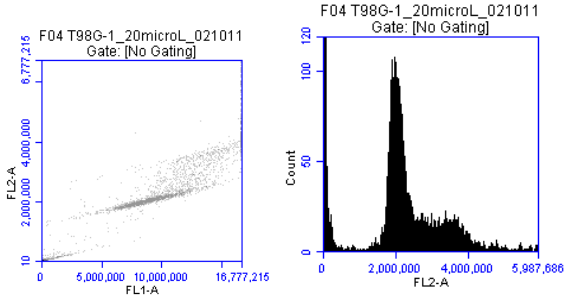


Figure A.2.7(C): *Plotsof DNA (FL2-A) verses Protein (FL1-A) and the DNA histogram before compensation with G1_CV =12.01.*

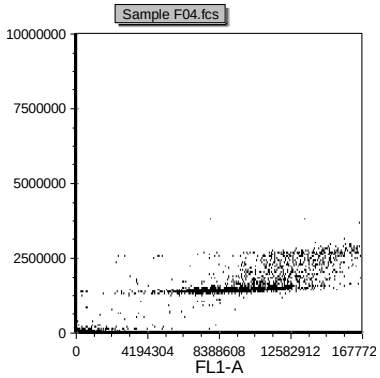


Figure A.2.7(D): *Plot of DNA (FL2-A) verses Protein (FL1-A) after compensation (8.0 %) with G1_CV =7.72.*

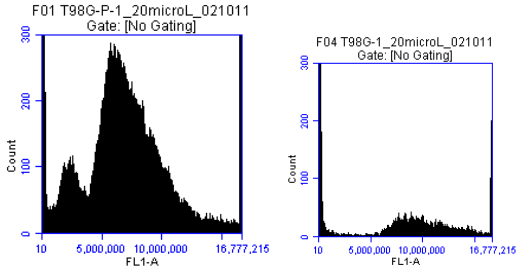


Figure A.2.7(E): *Protein (FL1-A) histograms for sample F01 with 50 % Rectified spirit and with FITC (0.1 µg/ml) in PBS (1X) and sample F04, T98G-P.*

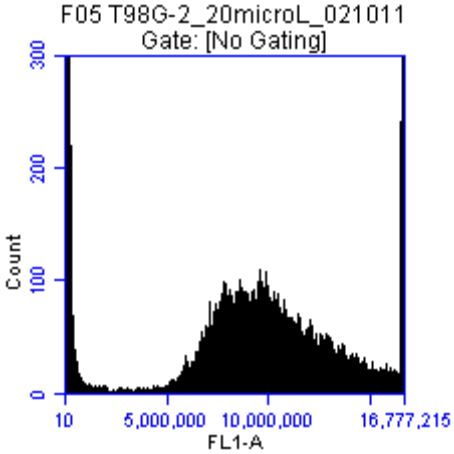


Figure A.2.7(F): *Protein (FL1-A) histogram for sample F05 with 50 % Rectified spirit and FITC (0.1µg/ml) in PBS (1X).*

Table A.2.6: *Cell Cycle distribution for T98G and T98G-P cells.*

Experiment 25			
Cell Type,	G1 %	S%	G2%
T98G 50 % Rectified Spirit -	58,31 ± 1,67	41,68 ± 1,67	00.00

FITC (0.1 µg/ml) in PBS (1X) – 3 samples			
T98G –P 50 % Rectified Spirit - FITC (0.1 µg/ml) in PBS (1X) -2 samples	60,98 ± 0,53	32,60 ± 2,09	6,41 ± 2,62

Photos	No photos

EXPERIMENT 26		
Date	07.10.2011 – 09.10.2011	EVENT(Cell) number :6000- 20 000
Used cell types and solutions with staining time		
Cell Type	T-47D	T-47D-P
Fixation of the cells	in 50 % PBS (1X) + 50 % Rectified Spirit in the freezer for 1 day	
First Staining	in FITC Solution - FITC (0.1 µg/ml) in PBS (1X), left overnight at room temperature in the dark	
Second Staining	1 hour in RNase Solution	
Third Staining	Minimum 1 hour in PI Solution	
Cleaning	with PBS (1X)	
The Results of the Experiment		
FCM		

BEFORE Compensation	AFTER Compensation

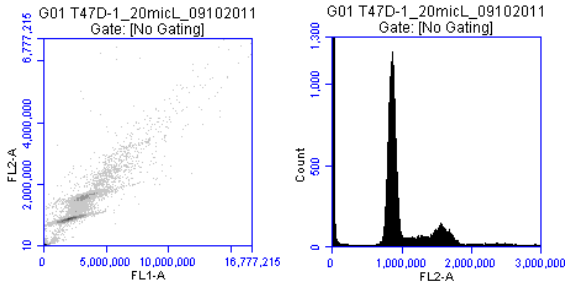
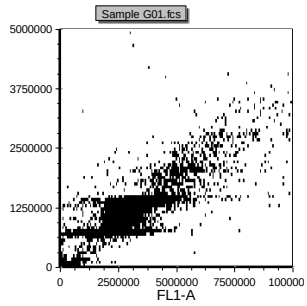

	

Figure A.2.8(A): *Plot of DNA (FL2-A) versus Protein (FL1-A) of T-47D before compensation with G1_CV =5.94 and its DNA histogram.*	***Figure A.2.8(B):*** *Plot of DNA (FL2-A) versus Protein (FL1-A) of T-47D after compensation (8 %) with G1_CV =3.88.*

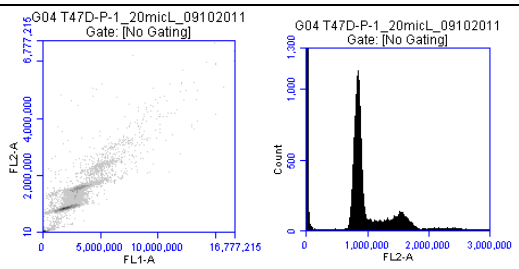


Figure A.2.8(C): *Plot of DNA (FL2-A) versus Protein (FL1-A) of T-47D-P and its DNA histogram before compensation with G1_CV =6.25.*

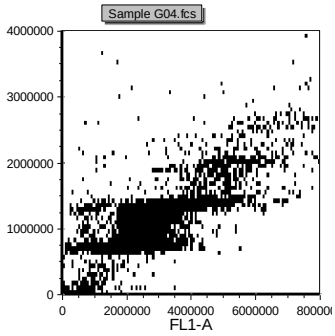


Figure A.2.8(D): *Plot of DNA (FL2-A) versus Protein (FL1-A) of T-47D –P after compensation (8.0 %) with G1_CV =4.28.*

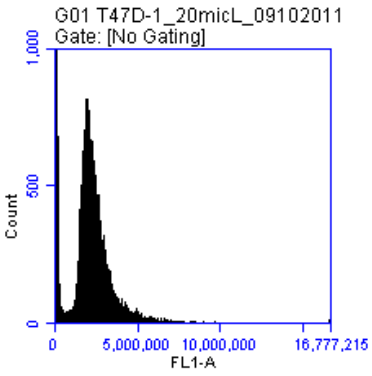


Figure A.2.8(E): *Protein (FL1-A) histogram of T-47D for sample G01 with 50 % Rectified spirit and with FITC (0.1 µg/ml) in PBS (1X).*

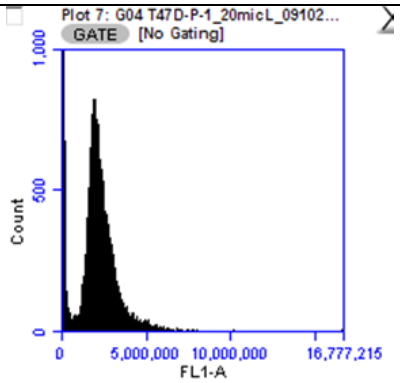


Figure A.2.8(F): *Protein (FL1-A) histogram of T-47-P for sample G04 with 50 % Rectified spirit and FITC (0.1µg/ml) in PBS (1X).*

Table A.2.7: *Cell Cycle distribution for T-47D and T-47D-P cells.*

Experiment 26			
Cell Type, 3 samples each	G1 %	S%	G2%
T-47D	68,54 ± 1,21	19,50 ± 1,33	11,94 ± 1,26
T-47D –P	67,76 ± 0,38	23,78 ± 3,16	8,45 ± 3,45

Photos	No photos

EXPERIMENT 27		
Date	14.10.2011 – 16.10.2011	EVENT(Cell) number :9000- 20 000
Used cell types and solutions with staining time		
Cell Type	T98G LDRres	
Fixation of the cells	in 50 % PBS (1X) + 50 % Rectified Spirit in the freezer for 1 day	
First Staining	in FITC Solution - FITC (0.1 µg/ml) in PBS (1X), left overnight at room temperature in the dark	
Second Staining	1 hour in RNase Solution	
Third Staining	Minimum 1 hour in PI Solution	
Cleaning	with PBS (1X)	
The Results of the Experiment		

FCM	
BEFORE Compensation	AFTER Compensation

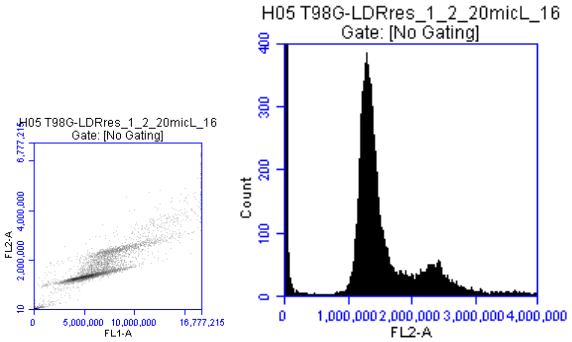


Figure A.2.9(A): *Plot of DNA (FL2-A) versus Protein (FL1-A) of T98G LDRres and its DNA histogram before compensation with G1_CV =8.91.*

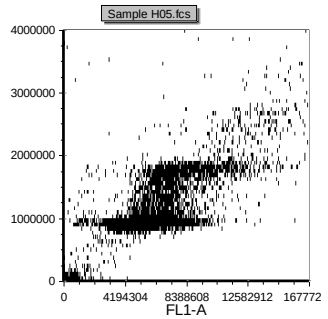


Figure A.2.9(B): *Plot of DNA (FL2-A) versus Protein (FL1-A) of T98G LDRres after compensation (8 %) with G1_CV =4.61.*

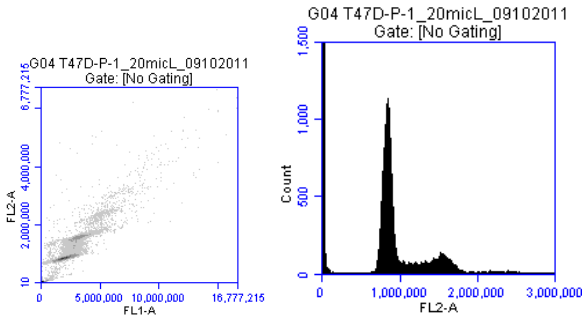


Figure A.2.9(C): *Plot of DNA (FL2-A) versus Protein (FL1-A) of T-47D-P before compensation with G1_CV =6.25 and its DNA histogram.*

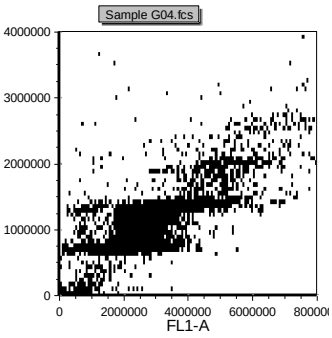


Figure A.2.9(D): *Plot of DNA (FL2-A) versus Protein (FL1-A) of T-47D –P after compensation (8.0 %) with G1_CV =4.28.*

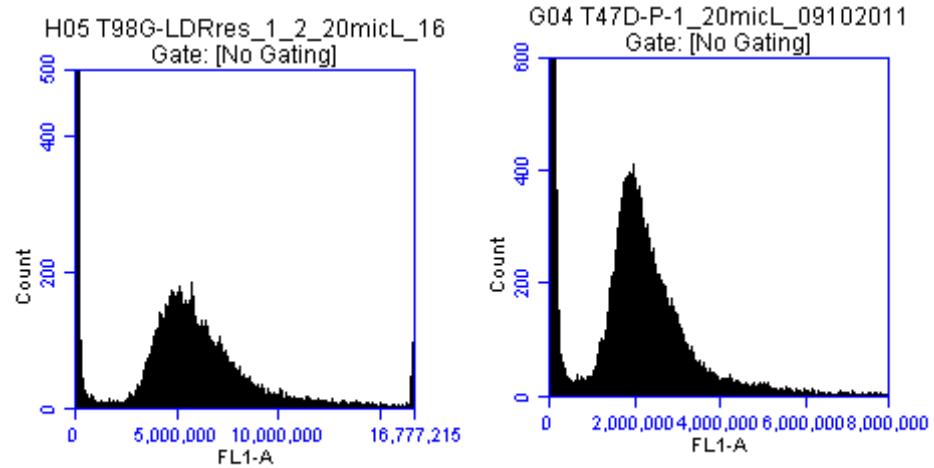


Figure A.2.9(E): *Protein (FL1-A) histograms of T98G LDRres for sample HO5 with 50 % Rectified spirit and with FITC (0.1 µg/ml) in PBS (1X) and of T47D-P cells.*

Table A.2.8: *Cell Cycle distribution for T-98G LDRres cells.*

Experiment 27

Cell Type, 4 samples	G1 %	S%	G2%
T98G LDRres	67,97 ± 0,48	21,40 ± 1,05	10,12 ± 0,82

Photos	No photos

EXPERIMENT 28		
Date	26.10.2011 – 28.10.2011	EVENT(Cell) number :5000- 10 000
Used cell types and solutions with staining time		
Cell Type	T98G LDRres	T98G
Fixation of the cells	in 50 % PBS (1X) + 50 % Rectified Spirit in the freezer for 1 day	
First Staining	in FITC Solution - FITC (0.1 µg/ml) in PBS (1X), left overnight at room temperature in the dark	
Second Staining	1 hour in RNase Solution	
Third Staining	Minimum 1 hour in PI Solution	
Cleaning	with PBS (1X)	
The Results of the Experiment		
FCM		

BEFORE Compensation

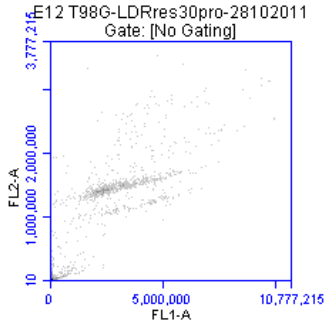


Figure A.2.10(A): *Plot of DNA (FL2-A) versus Protein (FL1-A) of T98G LDRres before compensation with G1_CV =7.16.*

AFTER Compensation

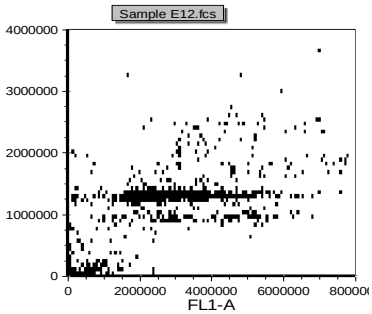
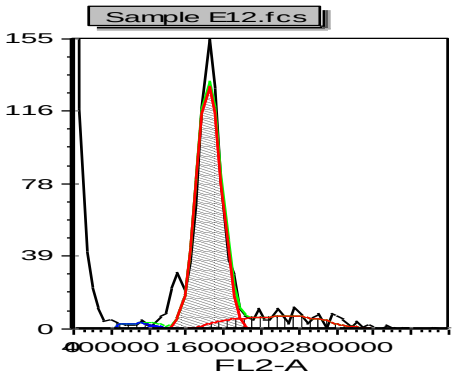


Figure A.2.10(B): *Plot of DNA (FL2-A) versus Protein (FL1-A) of T98G LDRres after compensation (7.5 %) with G1_CV =3.60.*



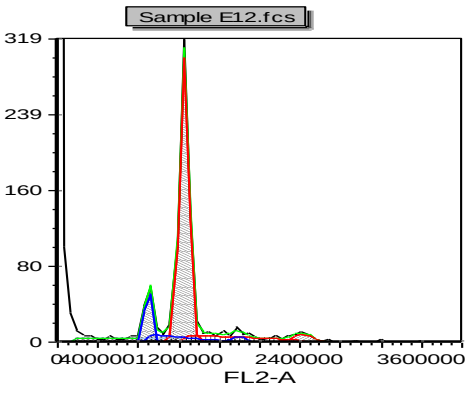


Figure A.2.10(C): *DNA (FL2-A) histogram for T98G and T98G LDRres cells before compensation with G1_CV =8.79, 7.16, respectively.*

Figure A.2.8(D): *DNA (FL2-A) histogram for T98G and T98G LDRres cells after compensation with G1_CV =3.60, 3.60, respectively.*

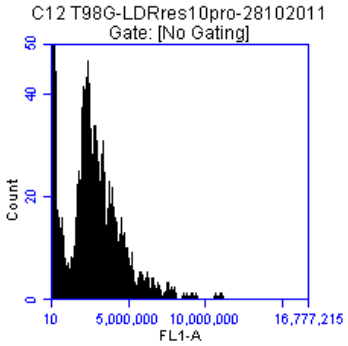


Figure A.2.10(E): *Protein (FL1-A) histogram of 90% T98G and 10 % T98G LDRres cells for sample C12.*

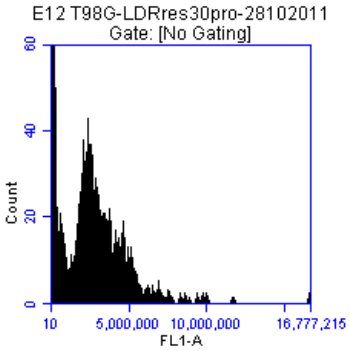


Figure A.2.10(F): *Protein (FL1-A) histogram of 70% T98G and 30 % T98G LDRres cells for sample E12.*

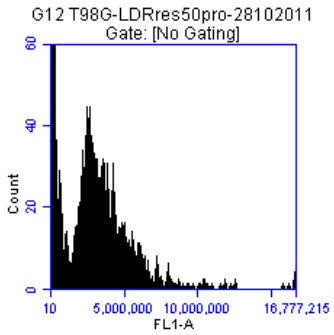


Figure A.2.10(G): *Protein (FL1-A) histogram of 50% T98G and 50 % T98G LDRres cells for sample G12.*

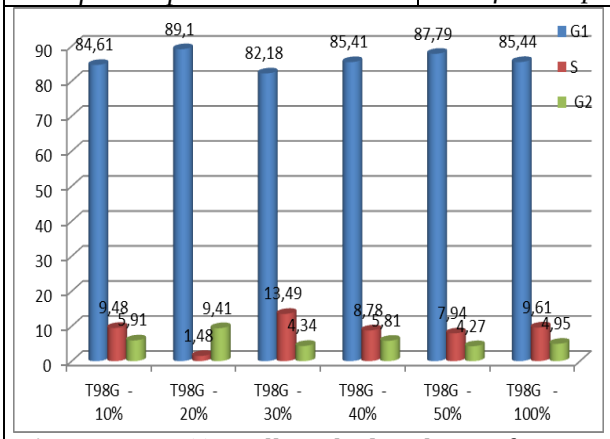


Figure A.2.10(I): *Cell Cycle distribution for 10% T98G -90% T98G LDRres, up to 50 % T98G LDRres -50 % T98G and 100 % T98G cells.*

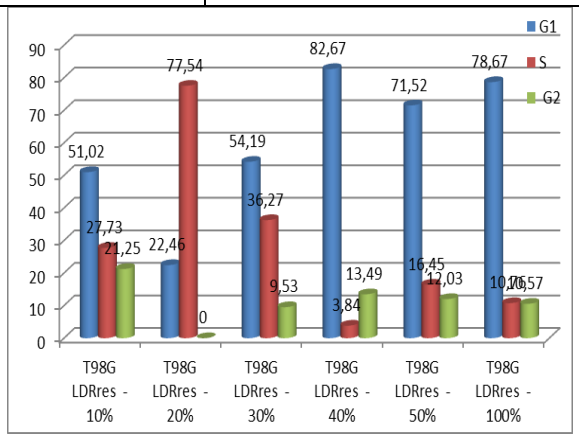


Figure A.2.10(I): *Cell Cycle distribution for 10% T98G LDRres -90% T98G, up to 50 % T98G LDRres -50 % T98G and 100 % T98G LDRres cells.*

Photos	No photos

Appendix B: The Compensation

In section 2.5.4.2, spectral compensation was discussed. In this appendix, the spectral compensation of one of the samples, which was performed using FCS Express software, will be presented. In addition to the compensation, how to create a DNA histogram and how to correct it manually are also presented.

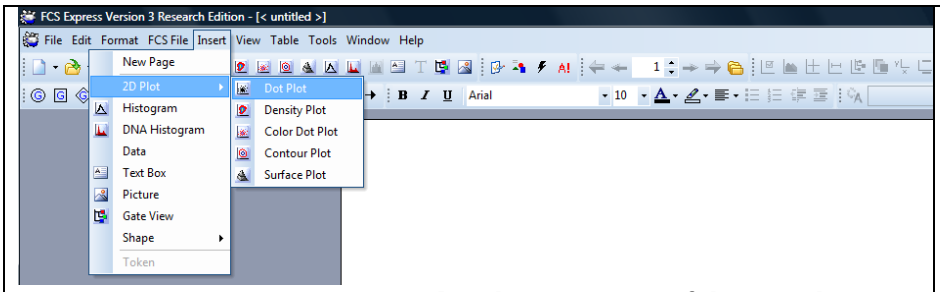


Figure B.1: *1. Step : Creating a dot plot using one of the samples, here sample A07.*

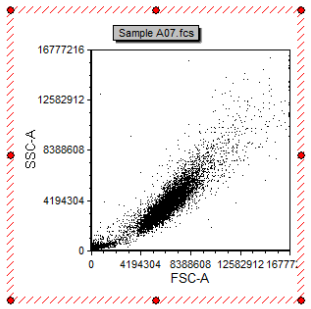


Figure B.2: *The result of the 1.step, the dot plot.*

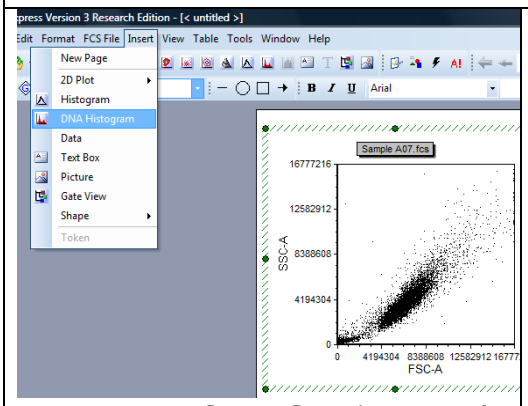


Figure B.3: *2. Step : Creating a DNA histogram for the sample A07.*

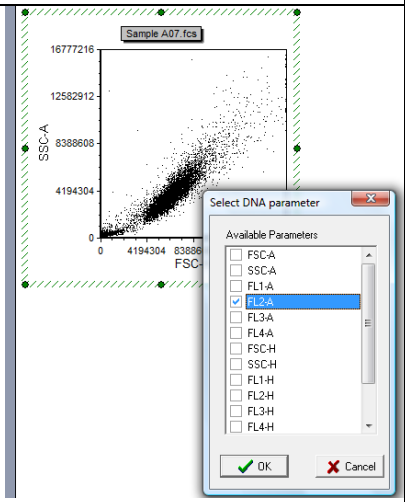


Figure B.4: *2. Step : Creating a DNA histogram for the sample A07.*

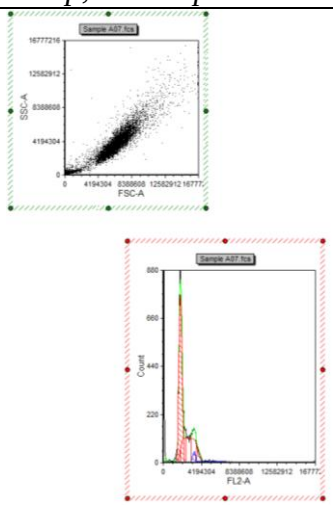


Figure B.5: *The result of the 2. Step, the DNA histogram.*

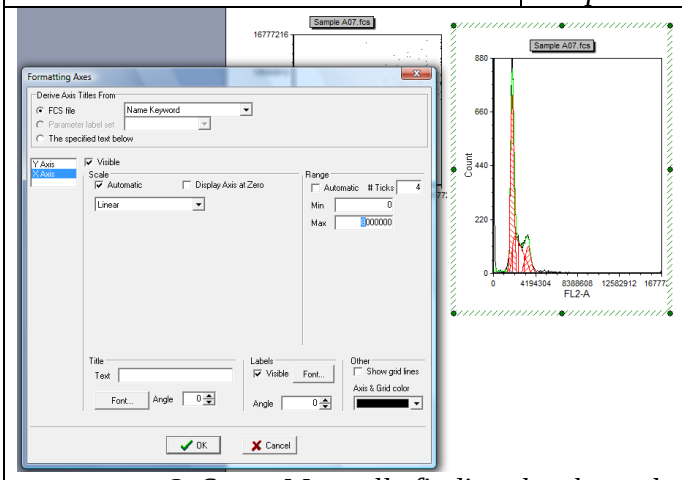


Figure B.6: *3. Step : Manually finding the channel peak number for G1.*

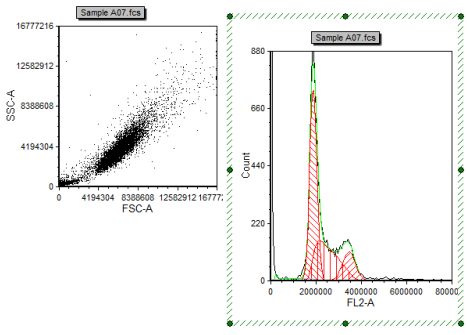


Figure B.7: *The result of 3.step, fixed DNA histogram still without compensation.*

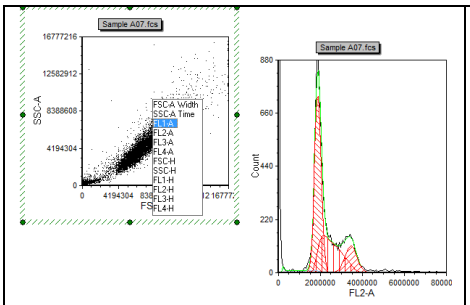


Figure B.8: *4. Step : Manually choosing Protein (FL1-A) and DNA (FL2-A) axes from the dot plot.*

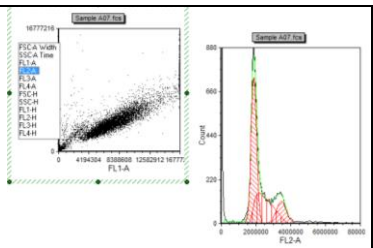


Figure B.9: *4. Step : Manually choosing Protein (FL1-A) and DNA (FL2-A) axes from the dot plot.*

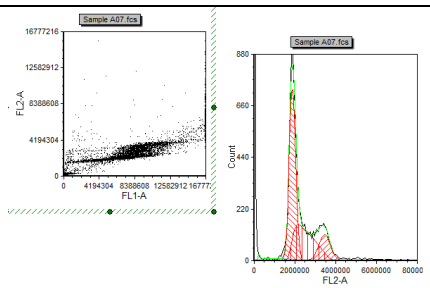


Figure B.10: The result of the *4. Step : the Protein (FL1-A) versus DNA (FL2-A) dot plot.*

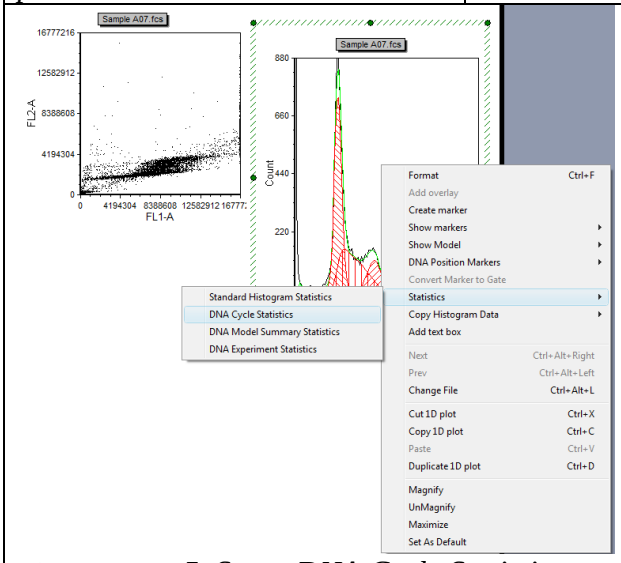


Figure B.11: *5. Step : DNA Cycle Statistics.*

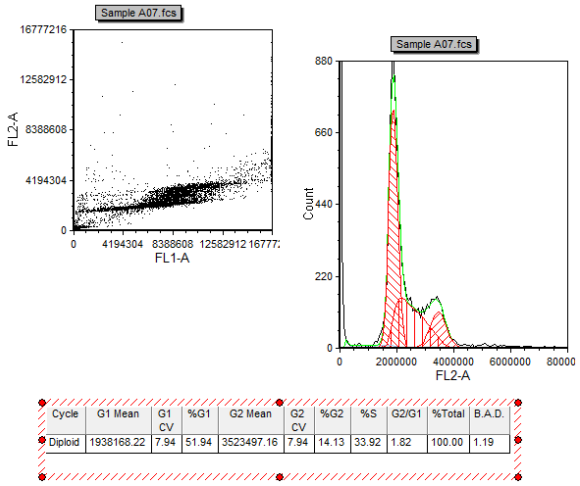


Figure B.12: The result of *5. Step, DNA Cycle Statistics still without compensation correction, G1_CV =7.94.*

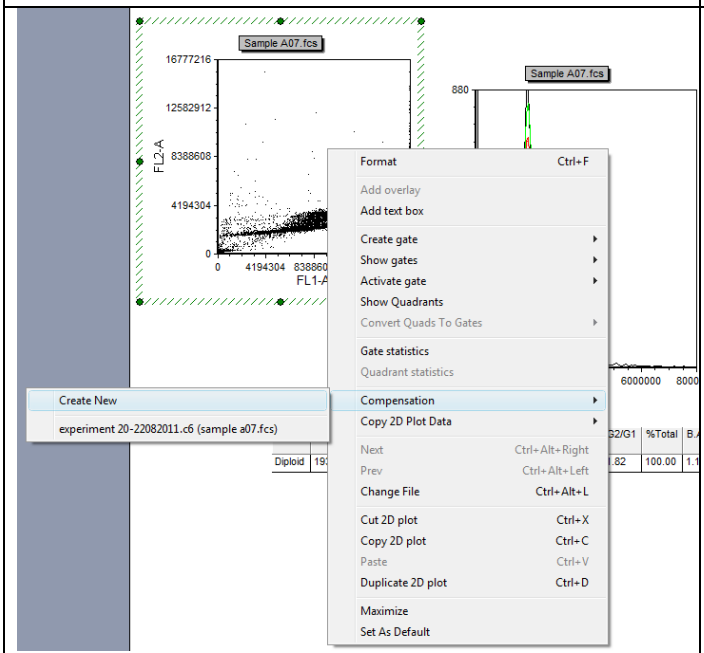


Figure B.13: *6. Step : Creating compensation.*

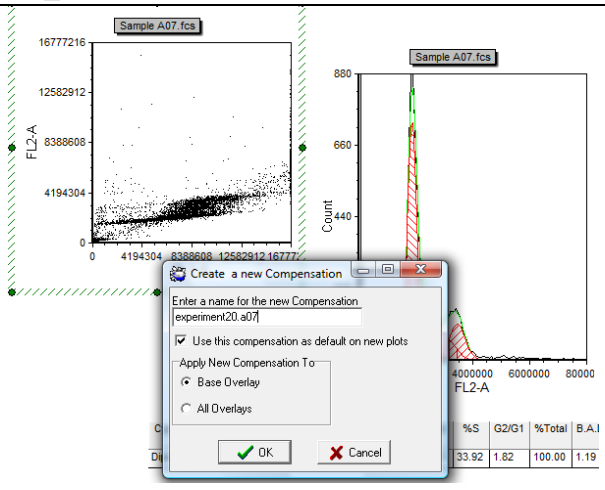


Figure B.14: *6. Step : Creating compensation, give a name for example experiment20 a07 then press OK.*

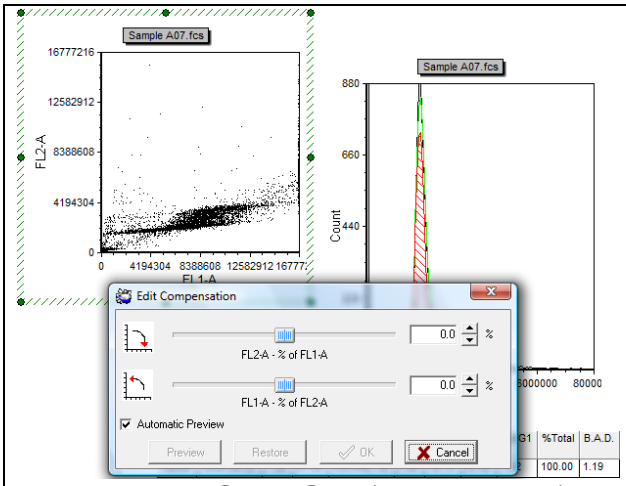


Figure B.15: *6. Step : Creating compensation, after OK, Edit compensation window pops up.*

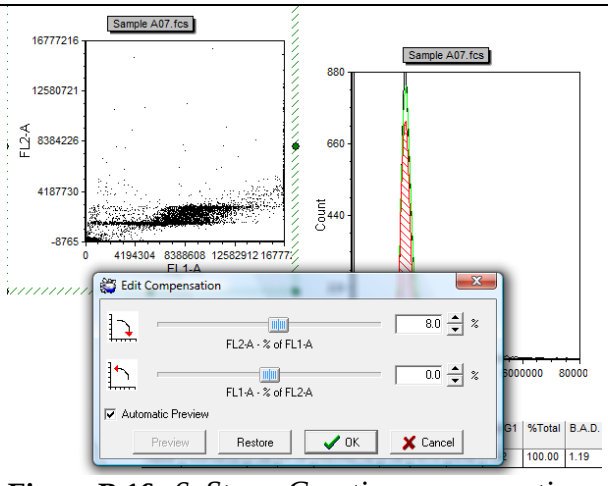
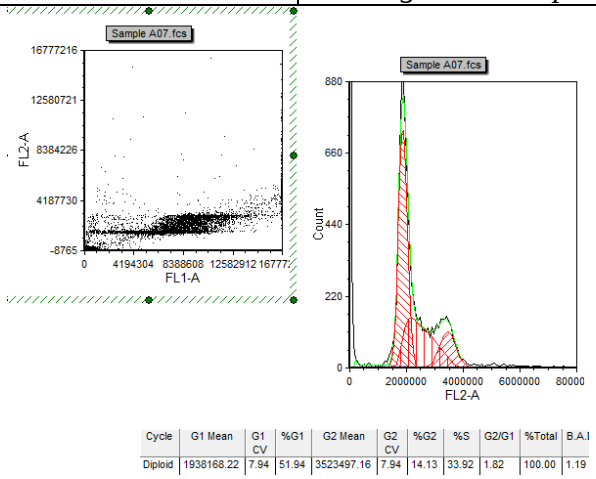


Figure B.16: *6. Step : Creating compensation, edit compensation while simultaneously looking at the dot plot.*



Cycle	G1 Mean	G1 CV	%G1	G2 Mean	G2 CV	%G2	%S	G2/G1	%Total	B.A.I
Diploid	1938168.22	7.94	51.94	3523497.16	7.94	14.13	33.92	1.82	100.00	1.19

Figure B.17: The result of the *6. Step, the compensated dot plot.*

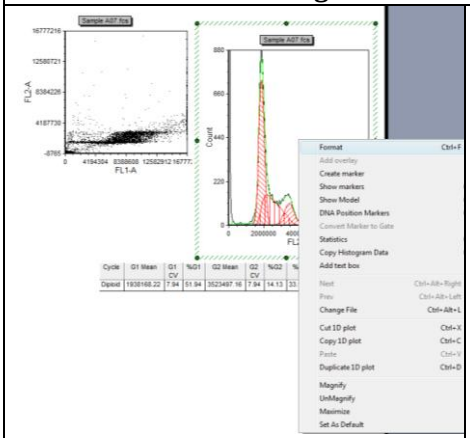


Figure B.18: *7. Step, applying the compensation to the DNA higtogram, click Format.*

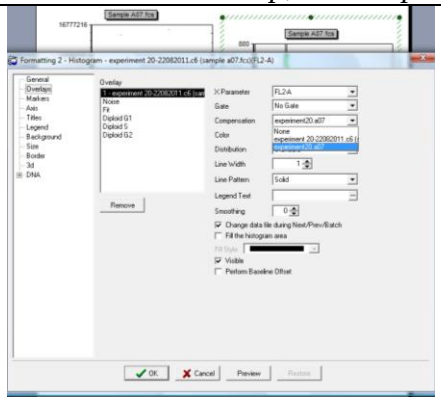


Figure B.19: *7. Step, applying the compensation to the DNA higtogram, choosing experiment20 a07 compensation then click OK.*

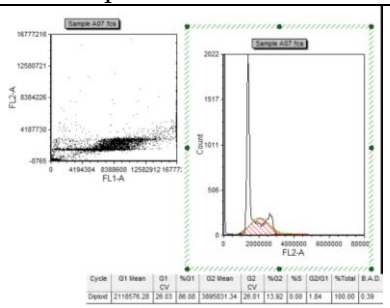


Figure B.20: The result of the *7. Step, the compensated DNA histogram.*

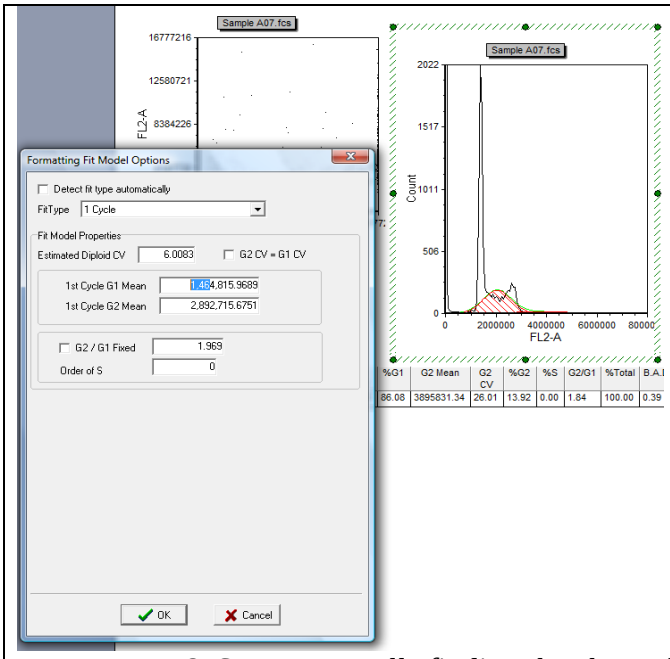


Figure B.21: *8. Step : Manually finding the channel peak number for G1for the compensated DNA histogram.*

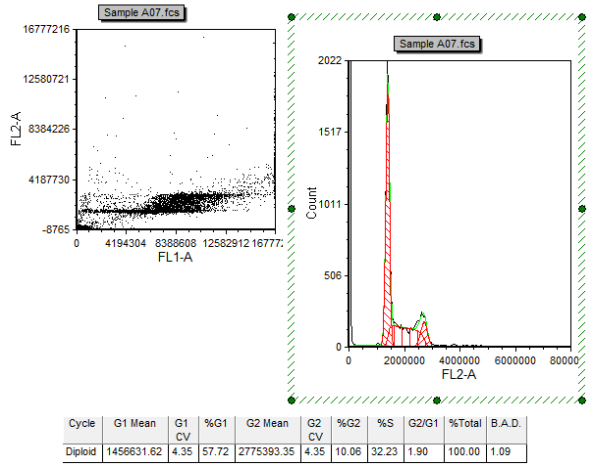


Figure B.22: The result of *8. Step: Compensated DNA histogram after manually finding the channel peak number for G1, with new G1_CV =4.35.*

Appendix C: History of Flow Cytometry

A brief history of flow cytometry can be summarized as following[27, 31, 32]:

1934	• Andrew Moldavan took first step from static microscopy toward a flowing system.
1954	• Wallace Coulter described an instrument in which an electronic measurement for cell counting and sizing was made on cells flowing in a conductive liquid with one cell at a time passing a measuring point. This became the basis of the first viable flow analyser.
1965	• A two-parameter flow cytometer that measured absorption and back-scattered illumination of unstained cells was described by Kamentsky et al. This was the first multiparameter flow cytometer and the same year the first cell sorter was described by Fulwyler. Use of an electrostatic deflection ink-jet recording technique enabled the instrument to sort cells in volume at a rate of 1000 cells per second.
1967	• Thompson developed a system for the electrostatic charging of droplets which enhanced the development of cell sorters. Van Dilla et al. exploited the real volume differences of cells to prepare suspensions of highly purified (>95%) human granulocytes and lymphocytes.
1969	• Dittrich and Göhde, in Germany, developed a fluorescence flow cytometer using arc-lamp epiillumination, the Imspulscytophotometer (ICP). They also introduced *ethidium bromide* as a stoichiometric fluorescent stain for DNA.
1970	• Phywe AG of Göttingen began selling a commercial version of the ICP, built around a Zeiss fluorescence microscope. United states were unaware of the existence of the ICP until 1973 or 1974.
1983	• First clinical flow cytometers were introduced.
1990	• Advances in technology, including availability of monoclonal antibodies (mAbs) and powerful but cheap computers, brought flow cytometry into routine use.
1995	• The ability to measure a minimum of five parameters on 25,000 cells in 1 second used routinely to enhance the diagnosis and management of various disease states.
1999	• Bigos et al. developed instruments equipped with lasers and capable of analysing 11 fluorochromes.
2003	• High-speed sorters using digital technology introduced.

Appendix D: List of some useful Fluorochromes

Fluorochrome	Absorption (nm)	Emission (nm)
Covalent labeling of proteins		
Fluorescein (FITC)	492	520–530
R-phycoerythrin (PE)	480–565	575–585
PerCP	490	677
Alexa 488	494	519
PE–Texas Red tandem	480–565	620
PE–Cy5 tandem	480–565	670
PE–Cy7 tandem	480–565	755
Tetramethylrhodamine (TRITC)	540	570
Cy3	540	567
Alexa 568	578	603
Texas Red	595	620
Cy5	648	670
Allophycocyanin	650	660
Nucleic acids		
Hoechst 33342 or 33258	346	460
DAPI	359	461
Chromomycin A3	445	575
Propidium iodide	480–580 (also UV)	623
Acridine orange		
+ DNA	480	520
+ RNA	440–470	650
Thiazole orange	509	525
7-aminoactinomycin D (7-AAD)	555	655
TO-PRO 3	642	661
Membrane potential		
$diOC_n(3)$ oxacarbocyanines	485	505
Rhodamine 123	511	546
pH		
Carboxyfluorescein		
High pH	495	520 (brt)
Low pH	450	520 (wk)
BCECF		
High pH	508	531 (brt)
Low pH	460–490	531 (wk)
Calcium		
Indo-1		
Low calcium	361	485
High calcium	330	405
Fluo-3		
High calcium	490	530 (brt)
Low calcium	490	530 (wk)
Reporter molecules		
E (enhanced) GFP	489	508
EBFP	380	440
ECFP	434	477
EYFP	514	527
DsRed	558	583
FDG (β-galactosidase substrate)	490	525
Cell tracking dyes		
CFSE	495	519
PKH 67	485	500
PKH 26	510 and 551	567

Figure 8.1: *Absorption and emission wavelength maxima of some useful fluorochromes[32].*

Appendix E: The Protocols in Details

Appendix E.1: The Vindeløv Method

1. *Picking the solutions up from the freezer*: The solutions A, B, C and citrate buffer (CB) are picked up from the – 80 degrees celcius freezer and inserted into the warm water bath. After the solutions are thawed, they and the 0.9 % NaCl salt are put into a good heat and light isolated box filled with ice + some water, +4 degrees celcius, the lid is closed.

2. *Trypsinization* : The medium of the cells is taken out, then 2 ml trypsin is inserted into the cell flask for 2-5 seconds. Take the trypsin out from the flask, insert 3 ml new trypsin into the flask, close the lid and put the flask into the incubator for 3 minutes.

3. *Making single cells* : Take the flask out, use suitable syringe and needle to convert the cells from colonies to single cells. Take the cells + the 3 ml trypsin out from the flask and insert into a tube filled with 3 ml medium.

4. *Cleaning*: Centrifuge the tube with 1100 rpm, 5 minutes and +4 degrees celcius. Suck the medium + trypsin. Insert 5 ml NaCI salt into the tube, vortex and centrifuge. Suck the salt, insert again 5 ml salt, vortex, centrifuge and suck the salt again. So you have cleaned the cells 2 times with NaCI. Now you have only the cells in the bottom of the tube.

5. *Adding solutions* :

    5.1. *Citrate Buffer (CB)*: Insert 500 µl citrate buffer into the tube, shake it by hand and use a pipette and cause the cells to be distributed homogenously in the CB. Wait 30 minutes at room temperature, keep the lid of the tube opened.

    5.2. *Solution A:* Insert 1.8 ml solution A into the tube, shake it by hand and use the pipette again. No need to heat, just room temperature. Total time after adding the solution A is 22 minutes both for T98G and T47D cell types.

Note: Use 0.5 ‰ Triton X-114 Solution A for T47D, 0.4 ‰ for T98G cell types.

    5.3. *Solution B*: Insert 1.5 ml solution B into the tube, shake it by hand and use the pipette. No heating, just room temperature. Total time after adding the solution B is 10 minutes.

    5.4. *Solution C*: Insert 1.5 ml solution C into the tube, shake it by hand and use the pipette. Put the tube into the ice + water box, close the lid and wait minimum 30 minutes. Avoid the cells with solution C and Solution C itself from light. Keep the lid closed.

6. Flow cytometry (FCM) measurements can be performed from 30 minutes to 3 hours after adding the solution C, when kept on ice and avoided light.

Appendix E.2: The two-parametric DNA- Protein Method

1. *Day 0 :*

Prepare a big flask of rectified spirit and put it into a freezer for minimum 1 day. You can use it again and again for the other experiments so the more you have it, the more you avoid "Day 1" step.

2. *Day 1 :*

2.1. *Trypsinization* : The medium of the cells is taken out, then 2 ml trypsin is inserted into the cell flask for 2-5 seconds. Take the trypsin out from the flask, insert 3 ml new trypsin into the flask, close the lid and put the flask into the incubator for 3 minutes.

2.2. *Making single cells*: Take the flask out, use suitable syringe and needle to convert the cells from colonies to single cells. Use the microscope to be sure if they are single cells. Take the cells + the 3 ml trypsin out from the flask and insert into a tube filled with 3 ml medium.

2.3. *Cleaning*: Centrifuge the tube(s) with 1100 rpm, 5 minutes and +4 degrees celcius. Suck the medium + trypsin. Insert 5 ml PBS (1X) into the tube(s), vortex and centrifuge. Suck the PBS, insert again 5 ml PBS, vortex, centrifuge and suck the PBS again. So you have cleaned the cells 2 times with PBS(1X). Now you have only the cells in the bottom of the tube.

2.4. Adding Rectified Spirit: After the cleaning, bring the rectified spirit from the freezer, put it into a box almost full with ice + some water. Insert slowly 2.5 ml PBS(1X) into each tube, then drop 2.5 ml rectified spirit (if you are slow, use 2.6 ml) into the tubes dropwise. The rectified spirit flask should be inside the ice + water box during the experiment. After the dropping, use a pipette to cause the cells and mixture to be distributed homogenously. Insert the tubes into the same freezer where you have your rectified spirit flask.

3. Day 2 :

3.1. Adding FITC: Take the tubes out from the freezer. Check if they are really frozen or still aqueous. They must be aqueous. Centrifuge them this time for 7 minutes, after the centrifugation you will notice that the cells are not really in the bottom of the tubes yet, so vortex them, then centrifuge for 5 minutes. Suck the PBS (1X) and the rectified spirit, clean with 1 times with 5 ml PBS (1X). Insert 3 ml FITC (0.1 µg/ml) in PBS (1X) into the tubes, vortex them gently, put them into a light and heat isolated box, close the lid until next day.

*4. **Day 3 :***

4.1 *Adding RNase:* Take the tubes out from the box, centrifuge them 5 minutes, suck the FITC, no cleaning with PBS (1X). Add 0.5 ml RNase (60 µg/ml) in PBS (1X) to each tube. Keep them in the box for 1 hour, the lid closed.

4.2 *Adding PI:* Insert 0.5 ml PI (34 µg/ml) into the tubes which had already 0.5 ml RNase. Make them wait in the box 1 hour, the lid closed.

4.3 *Flow Cytometry Measurements:* Flow cytometry measurements can be performed from 1 hour to 3 hours after the addition of PI, when kept on ice and avoided light.

Appendix F: Photos from T98G cell line

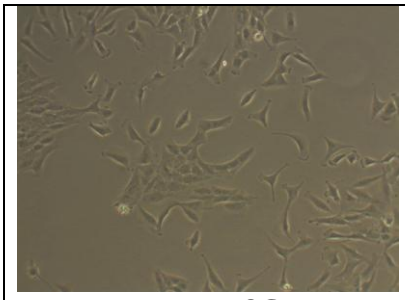


Figure F.1(A): *T98G-LDRres cells with LESS cell number/concentration.*

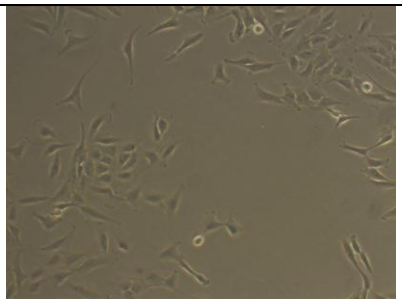


Figure F.1(B): *T98G-LDRres with cells LESS cell number/concentration.*

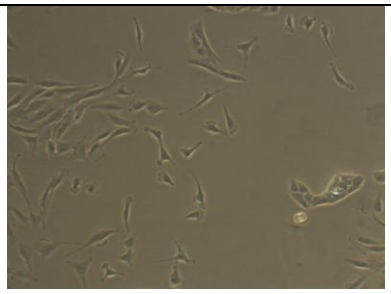


Figure F.1(C): *T98G-LDRres cells with LESS cell number/concentration.*

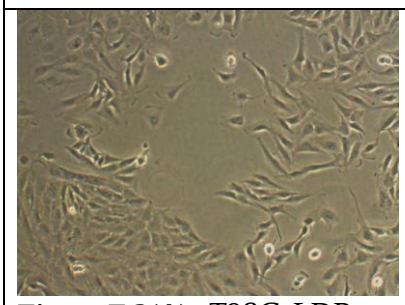


Figure F.2(A): *T98G-LDRres cells with MORE cell number/concentration.*

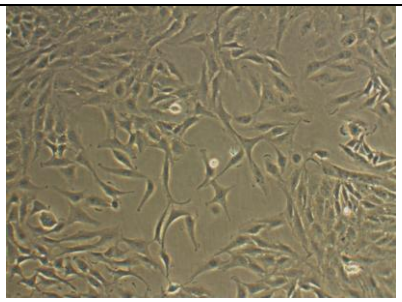


Figure F.2(B): *T98G-LDRres cells with MORE cell number/concentration.*

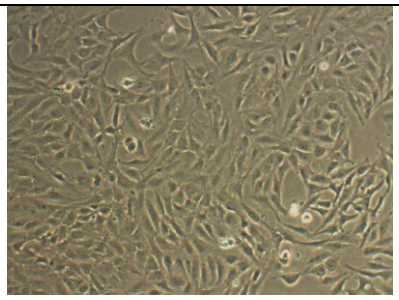


Figure F.2(C): *T98G-LDRres cells with MORE cell number/concentration.*

Figure F.3(A): *T98G cells with LESS cell number/concentration.*

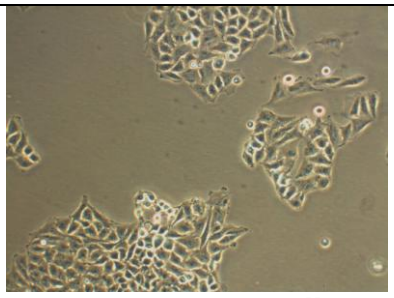


Figure F.3(B): *T98G cells with LESS cell number/concentration.*

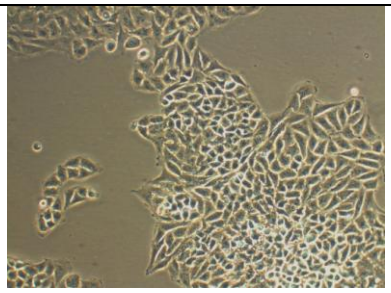


Figure F.3(C): *T98G cells with LESS cell number/concentration.*

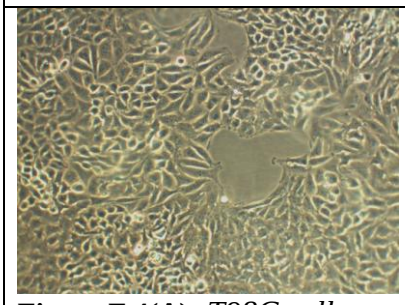


Figure F.4(A): *T98G cells with MORE cell number/concentration.*

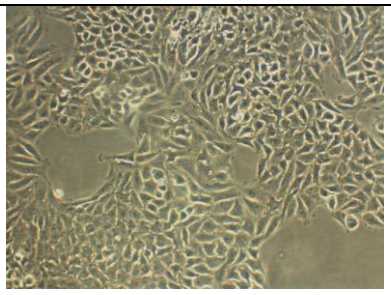


Figure F.4(B): *T98G cells with MORE cell number/concentration.*

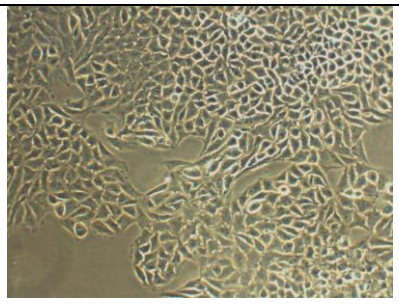
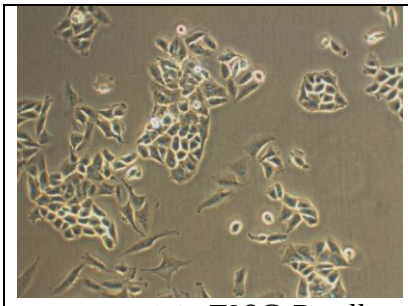
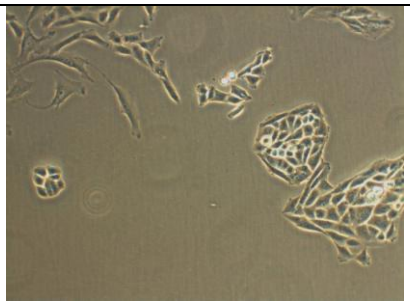
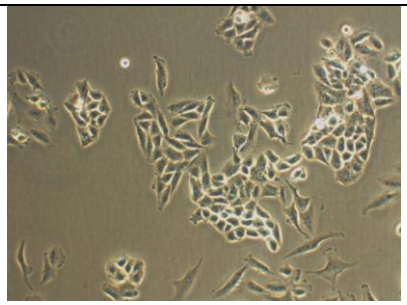
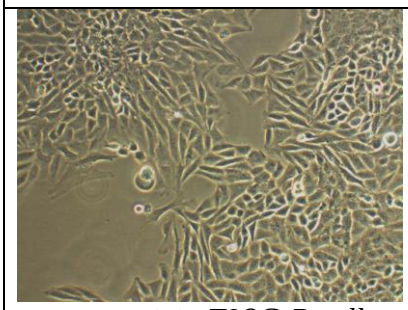
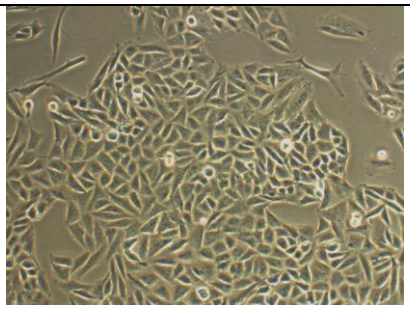
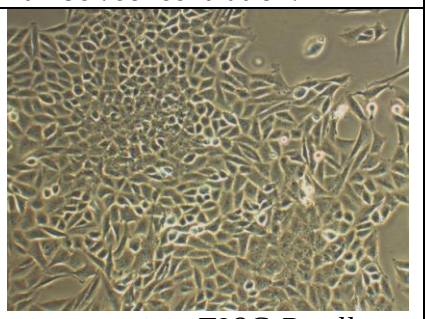


Figure F.4(C): *T98G cells with MORE cell number/concentration.*

 **Figure F.5(A):** *T98G-P cells with LESS cell number/concentration.*	 **Figure F.5(B):** *T98G-P cells with LESS cell number/concentration.*	 **Figure F.5(C):** *T98G-P cells with LESS cell number/concentration.*
 **Figure F.5(A):** *T98G-P cells with MORE cell number/concentration.*	 **Figure F.5(B):** *T98G-P cells with MORE cell number/concentration.*	 **Figure F.5(C):** *T98G-P cells with MORE cell number/concentration.*

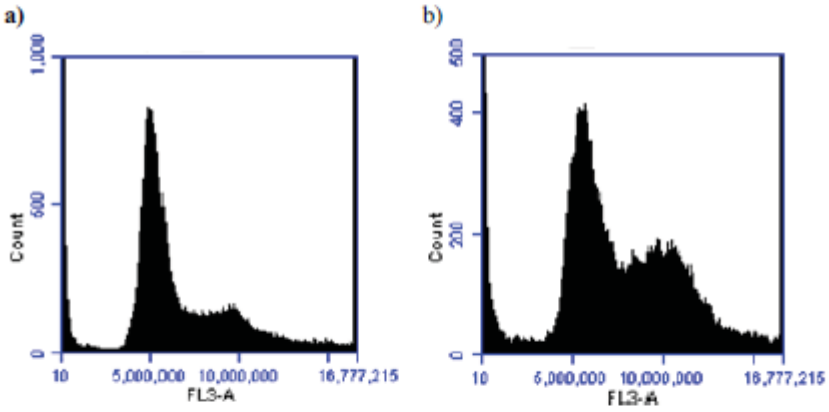
Appendix G: The Results of Ida Aspmodal (in Norwegian)

The Vindeløv method was used by Ida Aspmodal to apply it to the cells she was analyzing in her master project. Here are the relevant pages from her masther thesis.

4.6 Cellenes aldersfordeling

Det ble utført forsøk for å bestemme cellesyklusfordelingen etter at cellene hadde vokst på [^{3}H]-medium i 4 dager og i 11 dager. For cellene som hadde vokst på det radioaktive mediet med høyest spesifikk aktivitet (19.5 µCi/ml), ble det utført forsøk etter 4 og 7 dager.

Analysen av flowcytometridataene ble gjort med programmet FCS Express V3, og dot-plot og frekvenshistogrammer som ble brukt i analysen er lagt med i vedlegg G. Det er ikke benyttet noen form for gating på dot-plotene, så alle data er tatt med i analysen. Programvaren utfører automatisk 6 ulike variasjoner av analysen av cellesyklusfordelingen. Modell 6, CV's fixed, ble konsekvent benyttet for alle forsøkene som ble utført i denne oppgaven.



Figur 20: DNA-histogrammer for **a)** ubestrålte T98G-celler og **b)** T98G-celler bestrålt ved bruk av [^{3}H]-medium med spesifikk aktivitet på 19.5 µCi/ml, 4 dager ut i bestrålingsforløpet. DNA-histogrammene viser DNA-mengde langs x-aksen og relativt celleantall langs y-aksen.

Figur 20 viser DNA-histogrammer for T98G-celler som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 19.5 µCi/ml i 4 dager, i tillegg til DNA-histogrammer for ubestrålte T98G-celler. Fraksjonene av celler i de ulike delene av cellesyklus for de ubestrålte kontrollcellene og cellene som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 19.5 µCi/ml i hhv. 4 og 7 dager er vist i Tabell 11. For de bestrålte cellene hadde summen av dosen til cellekjernene nådd 59 Gy og 103 Gy etter hhv. 4 og 7 dager. De bestrålte cellene viser en økt fraksjon av celler i S-fase etter 4 dager i forhold til den ubestrålte kontrollen. Etter 7 dager er det en tydelig opphopning av celler i G_2-fasen for de bestrålte cellene, noe som kan sees ved at fraksjonen i G_2-fasen etter 7 dager for de bestrålte cellene er omtrent 4 ganger så stor som for de ubestrålte kontrollcellene.

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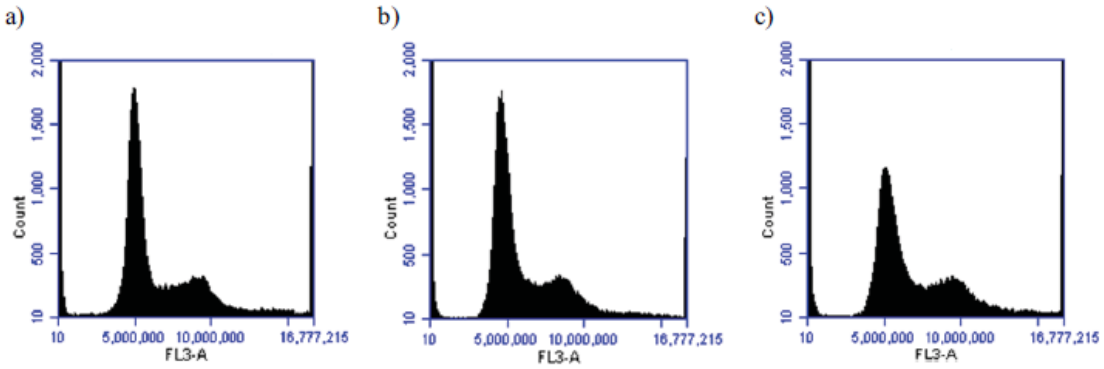
Tabell 11: Cellesyklusfordelingene for ubestrålte kontrollceller og celler bestrålt ved bruk av [^{3}H]-medium med spesifikk aktivitet på 19.5 µCi/ml hhv. **a)** 4 dager og **b)** 7 dager ut i bestrålingsforløpet.

a)

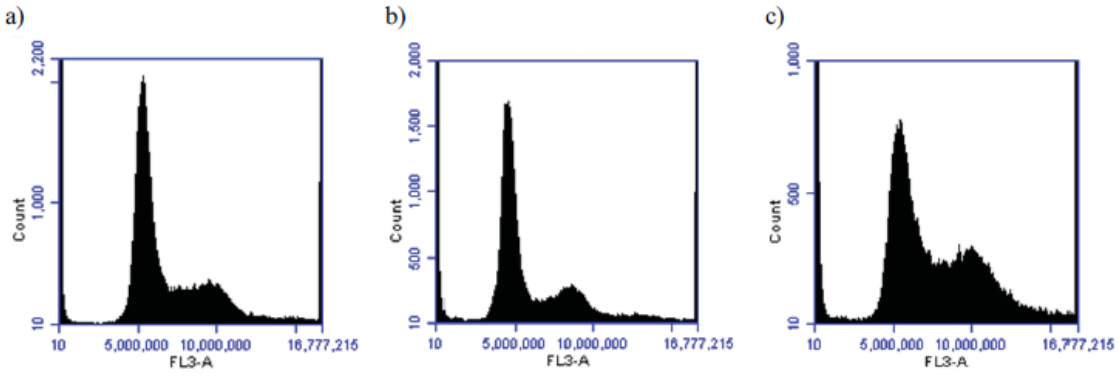
Dose (µCi/ml)	% G_1	% S	% G_2
Kontroll	49.30	45.53	5.17
19.5	34.99	58.87	6.14

b)

Dose (µCi/ml)	% G_1	% S	% G_2
Kontroll	45.11	50.23	4.67
19.5	51.58	30.22	18.20



Figur 21: DNA-histogrammer for a) ubestrålte T98G-celler, b) T98G-celler bestrålt ved bruk av [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml og c) T98G-celler bestrålt ved bruk av [^{3}H]-medium med spesifikk aktivitet på 6.5 µCi/ml, 4 dager ut i bestrålingsforløpet. DNA-histogrammene viser DNA-mengde langs x-aksen og relativt celleantall langs y-aksen.



Figur 22: DNA-histogrammer for a) ubestrålte T98G-celler, b) T98G-celler bestrålt ved bruk av [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml og c) T98G-celler bestrålt ved bruk av [^{3}H]-medium med spesifikk aktivitet på 6.5 µCi/ml, 11 dager ut i bestrålingsforløpet. DNA-histogrammene viser DNA-mengde langs x-aksen og relativt celleantall langs y-aksen.

Figur 21 og Figur 22 viser DNA-histogrammer for T98G-celler som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 og 6.5 µCi/ml i hhv. 4 og 11 dager, i tillegg til DNA-histogrammer for ubestrålte T98G-celler. Fraksjonene av celler i de ulike delene av cellesyklus for de ubestrålte kontrollcellene og cellene som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 og 6.5

µCi/ml i hhv. 4 og 11 dager er vist i Tabell 12. For cellene som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml hadde summen av dosen til cellekjernene nådd 5 Gy og 14 Gy etter hhv. 4 og 11 dager, mens summen av dosen til cellekjernene for cellene som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 6.5 µCi/ml hadde nådd 20 Gy og 54 Gy etter hhv. 4 og 11 dager. Cellene som er bestrålt med den laveste doseraten viser ingen stor endring i cellesyklusfordelingen i forhold til den ubestrålte kontrollen. For cellene som er bestrålt ved bruk av [^{3}H]-medium med spesifikk aktivitet på 6.5 µCi/ml ses en endring i cellesyklusfordelingen i forhold til den ubestrålte kontrollen, ved at fraksjonen av celler i S-fase er økt og ved en opphopning av celler i G_2-fasen for de bestrålte cellene. Fraksjonen av celler i G_2-fasen etter 7 dager er nesten doblet for cellene som er bestrålt med den høyeste doseraten i forhold til de ubestrålte kontrollcellene.

Tabell 12: Cellesyklusfordelingene for ubestrålte T98G-celler og T98G-celler bestrålt ved bruk av [^{3}H]-medium med spesifikk aktivitet på 1.67 og 6.5 µCi/ml hhv. a) 4 dager og b) 11 dager ut i bestrålingsforløpet.

a)

Dose (µCi/ml)	% G_1	% S	% G_2
Kontroll	50.51	45.82	3.67
1.67	50.97	46.32	2.71
6.5	44.11	52.12	3.78

b)

Dose (µCi/ml)	% G_1	% S	% G_2
Kontroll	50.69	44.50	3.81
1.67	56.91	38.18	4.91
.5	34.99	57. 4	7.37

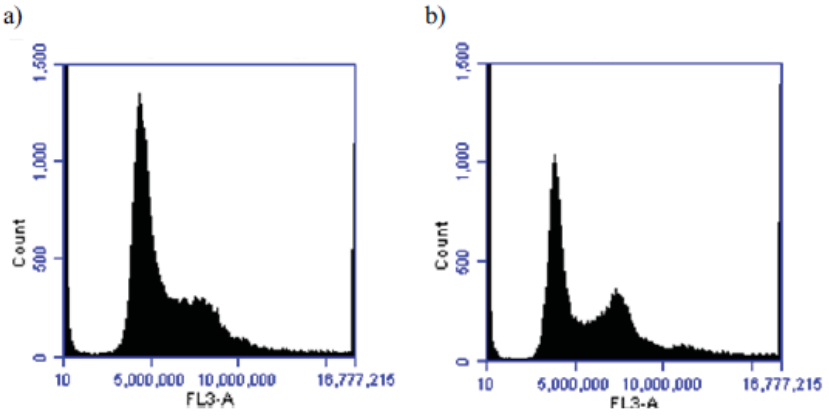
Tabell 13: Cellesyklusfordelingen for ubestrålte T98G-celler og T98G-celler bestrålt ved bruk av [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml 195 dager ut i bestrålingsforløpet.

Dose (µCi/ml)	% G_1	% S	% G_2
Kontroll	44.12	51.45	4.44
1.67	45.53	38.69	17.78

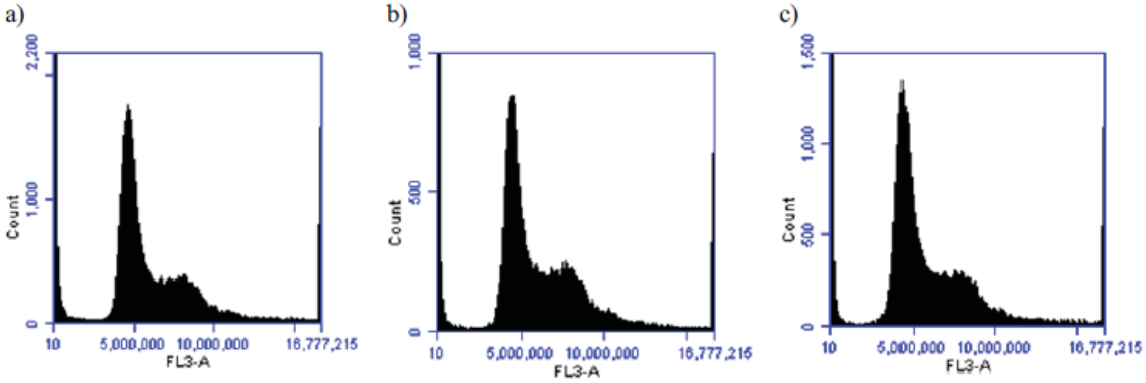
Figur 23 viser DNA-histogrammer for ubestrålte T98G-celler og T98G-celler som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 195 dager. Fraksjonen av celler i de ulike delene av cellesyklus for de ubestrålte T98G-cellene og cellene som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 195 dager er vist Tabell 13. For de bestrålte cellene hadde summen av dosen til cellekjernene nådd omtrent 246 Gy ved gjennomføring av forsøket. De bestrålte cellene viser en tydelig endring i cellesyklusfordelingen i forhold til de ubestrålte cellene, ved at fraksjonen av celler i G_2-fasen for de bestrålte T98G-cellene er omtrent 4 ganger så stor som for de ubestrålte T98G-cellene.

Figur 24 viser DNA-histogrammer for T98G-celler som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 0.28 og 0.53 µCi/ml i 4 dager, i tillegg til DNA-histogrammer for ubestrålte T98G-celler. Fraksjonene av celler i de ulike delene av cellesyklus for de ubestrålte kontrollcellene og cellene som

hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 0.28 og 0.53 µCi/ml er vist i Tabell 14. For de bestrålte cellene hadde summen av dosen til cellekjernene ved gjennomføring av forsøket nådd omtrent 0.84 Gy og 1.68 Gy. De bestrålte T98G-cellene viser ingen særlig endring i cellesyklusfordelingen i forhold til de ubestrålte T98G-cellene.



Figur 23: DNA-histogrammer for **a)** ubestrålte T98G-celler og **b)** T98G-celler bestrålt ved bruk av [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml, 195 dager ut i bestrålingsforløpet. DNA-histogrammene viser DNA-mengde langs x-aksen og relativt celleantall langs y-aksen.



Figur 24: DNA-histogrammer for **a)** ubestrålte T87G-celler, **b)** T98G-celler bestrålt ved bruk av [^{3}H]-medium med spesifikk aktivitet på 0.28 µCi/ml og **c)** T98G-celler bestrålt ved bruk av [^{3}H]-medium med spesifikk aktivitet på 0.53 µCi/ml, 4 dager ut i bestrålingsforløpet. DNA-histogrammene viser DNA-mengde langs x-aksen og relativt celleantall langs y-aksen.

Det ble i tillegg utført flowcytometrimålinger på cellekjerner preparert ved bruk av Vindelövs metode, etablert og utført av Celal Ceyhan (Ceyhan 2011). Analysen av flowcytometridataene ble gjort med programmet FCS Express V3. Det er ikke benyttet noen form for gating på dot-plotene, så alle data er tatt med i analysen. Flowcytometrimålingene ble utført på vanlige T98G-celler og T-47D-celler, i tillegg til T98G-celler som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder. Koloniforsøk utført på cellene som var lavdoseratebestrålt ved bruk av [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml viste at overlevelsesfraksjonen, etter å ha vært nede på rundt 0.4 % etter omtrent 100 dager, steg og stabiliserte seg på rundt 50-60 % relativt til ubestrålte T98G-celler

mot slutten av forsøket (se kapittel 4.5). Det var derfor interessant å undersøke om disse cellene viste en endring i cellesyklusfordelingen i forhold til ubestrålte T98G-celler.

Tabell 14: Cellesyklusfordelingene for ubestrålte T98G-celler og T98G-celler bestrålt ved bruk av [^{3}H]-medium med spesifikk aktivitet på 0.28 og 0.53 µCi/ml 4 dager ut i bestrålingsforløpet.

Dose (µCi/ml)	% G_1	% S	% G_2
Kontroll	45.95	50.15	3.90
0.28	39.05	56.93	4.02
0.53	41.63	55.82	2.55

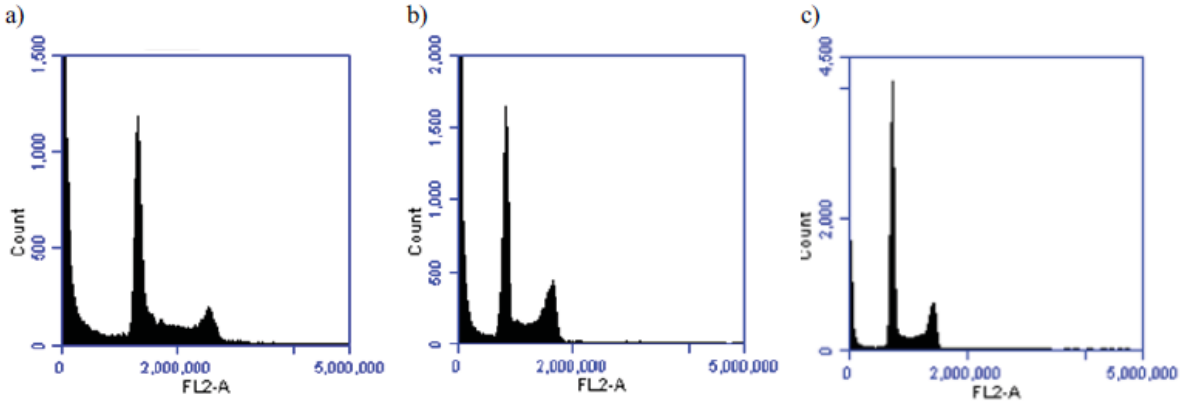
Tabell 15 viser den gjennomsnittlige fraksjonen av cellekjerner i hver av fasene i cellesyklus for hhv. T98G-celler, T98G-celler som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder og T-47D-celler. T98G-cellene som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder skiller seg noe fra de to andre celletypene ved at fraksjonen av cellekjerner i S-fasen er høyere. Andelen cellekjerner i G_2-fasen er omtrent lik for de tre celletypene.

Tabell 15: Cellesyklusfordelingene for T98G-celler, T98G-celler som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder og T-47D-celler, etter flowcytometriforsøk utført på cellekjerner preparert ved bruk av Vindelövs metode.

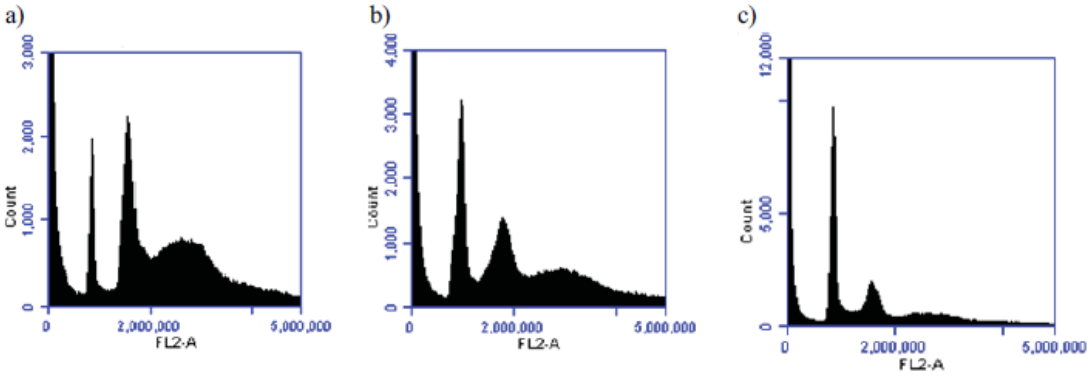
Celletype	% G_1	% S	% G_2
T98G	49.21 ± 2.74	38.16 ± 4.03	12.63 ± 1.39
T98G lavdoseratebestrålt	33.52 ± 0.08	53.58 ± 1.02	13.00 ± 0.94
T-47D	55.56 ± 0.90	30.99 ± 0.79	13.45 ± 0.11

Figur 25 a) - c) viser DNA-histogrammer for hhv. T98G-kjerner, kjerner fra T98G-celler som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder og T-47D-kjerner. Det er en forskjell i fluorescensintensiteten hos T98G-kjernene sammenliknet med T-47D-kjernene, som skyldes ulikt DNA-innhold i cellekjernen hos T98G- og T-47D-celler. G_1-toppen i DNA-histogrammet for T-47D-kjernene kommer lenger til venstre på x-aksen enn for T98G-kjernene, noe som kan tyde på et lavere DNA-innhold for T-47D enn for T98G. Figur 25 b) viser at det også er en forskjell i fluorescensintensiteten for vanlige T98G-kjerner og kjernene fra T98G-cellene som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder før flowcytometriforsøkene ble gjennomført. Det ser ut til at DNA-innholdet i kjernen til de lavdoseratebestrålte T98G-cellene er lavere enn for de ubestrålte T98G-cellene. Forskjellen i fluorescensintensitet kan tyde på en forskjell i DNA-innhold hos de forskjellige celletypene, men kan også indikere forskjell i DNA-fargingen ved inkorporering av PI. Det ble derfor utført et flowcytometriforsøk på celleprøver som besto av

ubestrålte T98G-kjerner og kjerner fra T98G-celler som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder, blandet sammen.



Figur 25: DNA-histogrammer for **a)** T98G-kjerner, **b)** kjerner fra T98G-celler som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder og **c)** T-47D-kjerner. DNA-histogrammene viser DNA-mengde langs x-aksen og relativt kjerneantall langs y-aksen.



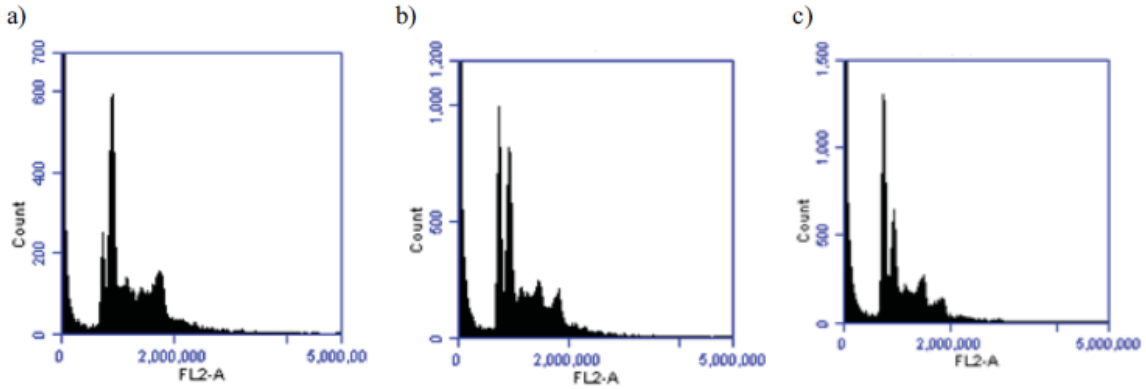
Figur 26: DNA-histogrammer for prøver som består av **a)** 90 % ubestrålte T98G-kjerner og 10 % T98G-kjerner som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder, **b)** 70 % ubestrålte T98G-kjerner og 30 % T98G-kjerner som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder og **c)** 50 % ubestrålte T98G-kjerner og 50 % T98G-kjerner som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder. DNA-histogrammene viser DNA-mengde langs x-aksen og relativt kjerneantall langs y-aksen.

Figur 26 viser DNA-histogrammer for flowcytometriforsøk utført på prøver som besto av både ubestrålte T98G-kjerner og kjerner fra T98G-celler som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder, blandet sammen. Figur 26 a), b) og c) viser DNA-histogrammet for en prøve som inneholdt hhv. 90 % T98G-kjerner og 10 % T98G-kjerner som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder, 70 % T98G-kjerner og 30 % T98G-kjerner som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder, og 50 % T98G-kjerner og 50 % T98G-kjerner som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder. Alle prøvene ble preparert samtidig og på samme måte, og det skal derfor ikke være noen forskjell i DNA-fargingen. Forskjellen i fluorescensintensitet tyder dermed på at DNA-

innholdet i kjernen er forskjellig for de ubestrålte T98G-cellene og T98G-cellene som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder.

Det lavere DNA-innholdet i kjernen til de lavdoseratebestrålte T98G-cellene gav en mistanke om at det en gang i løpet av bestrålingsforløpet hadde skjedd en forurensing av celleprøvene, slik at T-47D-celler hadde blitt blandet sammen med de lavdoseratebestrålte T98G-cellene. For å utelukke at dette hadde skjedd, ble det utført et flowcytometriforsøk på celleprøver som besto av både T47D-celler og T98G-celler som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder for å sammenlikne DNA-innholdet til de to celletypene.

Figur 27 viser DNA-histogrammer for flowcytometriforsøk utført på prøver som besto av både T-47D-celler og T98G-celler som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder blandet sammen. Figur 27 a), b) og c) viser DNA-histogrammet for en prøve som inneholdt hhv. 10 % T-47D-kjerner og 90 % T98G-kjerner som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder, 30 % T-47D-kjerner og 70 % T98G-kjerner som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder og 50 % T-47D-kjerner og 50 % T98G-kjerner som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder. Det er en tydelig forskjell mellom G_1 - og G_2 - toppene til de to ulike celletypene, noe som igjen betyr at DNA-innholdet i cellekjernen for de to celletypene er ulikt.



Figur 27: DNA-histogrammer for prøver som består av **a)** 10 % T-47D-kjerner og 90 % T98G-kjerner som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder, **b)** 30 % T-47D-kjerner og 70 % T98G-kjerner som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder og **c)** 50 % T-47D-kjerner og 50 % T98G-kjerner som hadde vokst på [^{3}H]-medium med spesifikk aktivitet på 1.67 µCi/ml i 10 måneder. DNA-histogrammene viser DNA-mengde langs x-aksen og relativt kjerneantall langs y-aksen.

Appendix H: Error Interval of G1_CV values due to Software Problem

To analyse the data, FSC Express Software was used. The Software could not automatically find correct G1 peak values/channel numbers therefore it had to be found manually. Three samples/dot plots were investigated to find the upper and lower channel numbers which result different G1_ CV values.

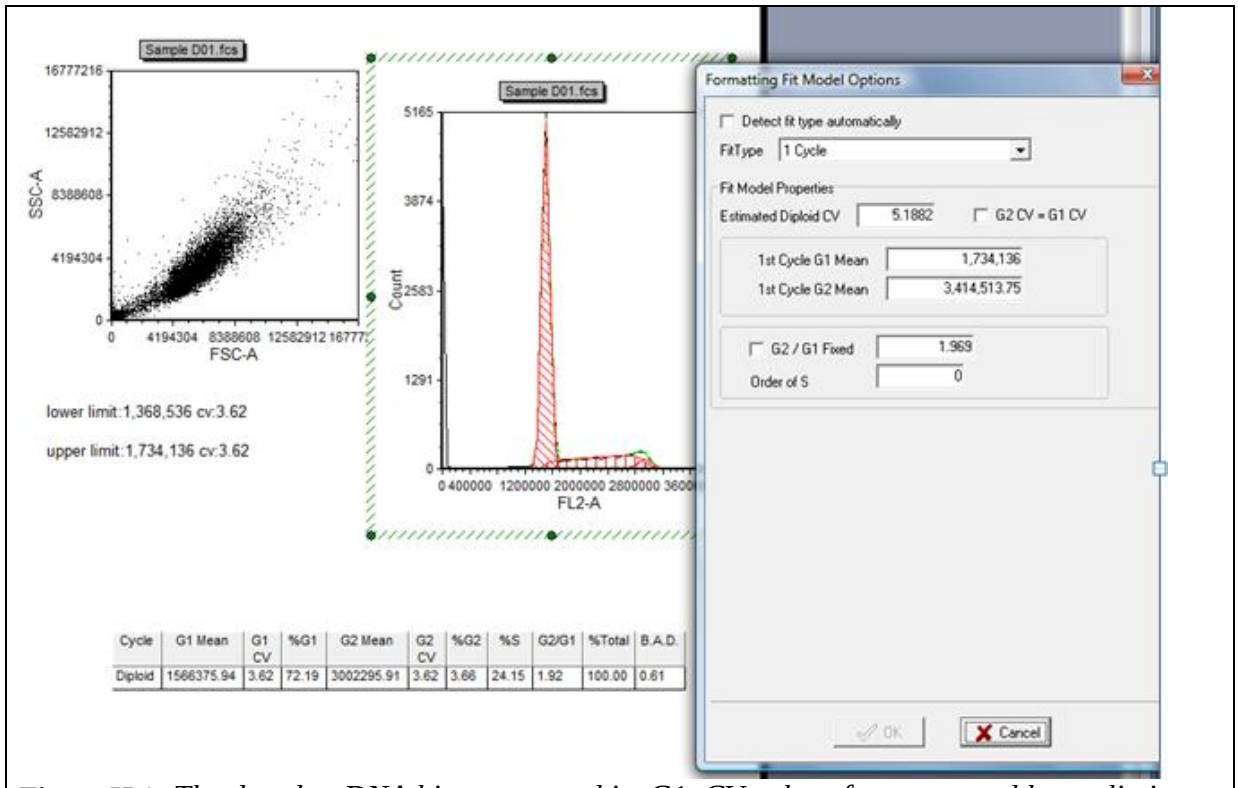


Figure H.1: *The dot plot, DNA histogram and its G1_CV values for upper and lower limits, and Formatting fit type Model Options window for sample D01 in experiment 23.*

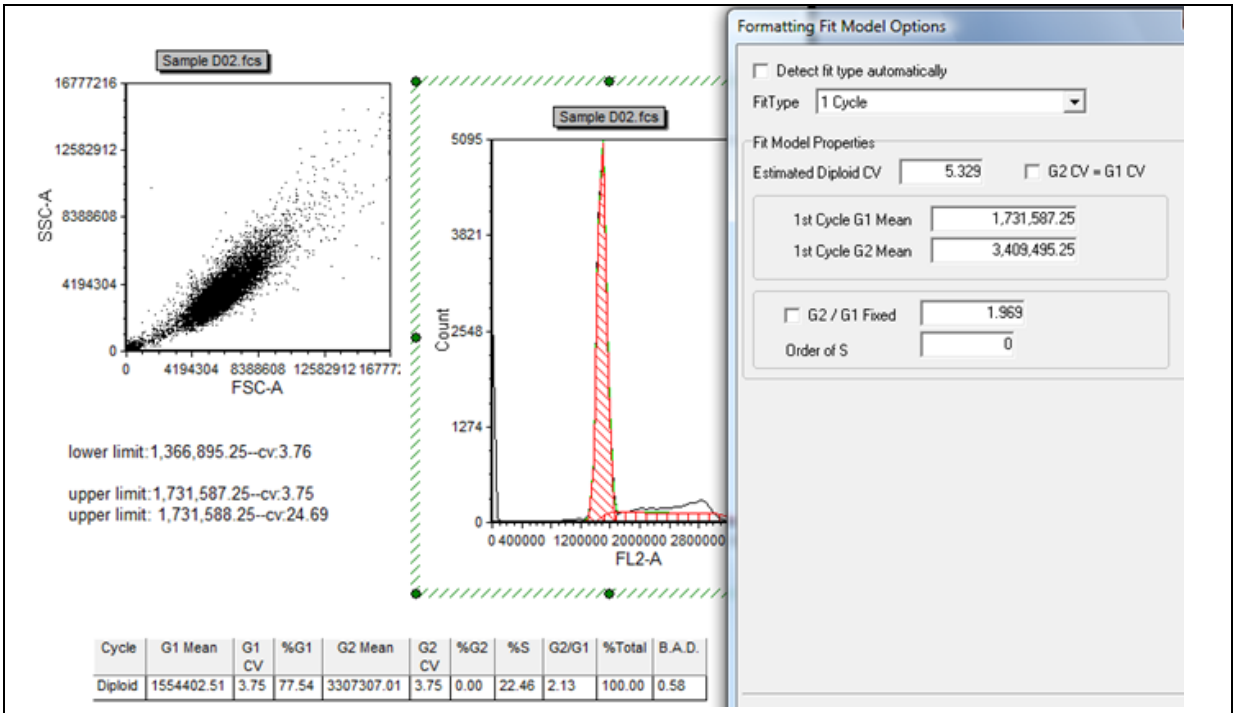


Figure H.2: *The dot plot, DNA histogram and its G1_CV values for lower and upper limits, and Formatting fit type Model Options window for sample D02 in experiment 23.*

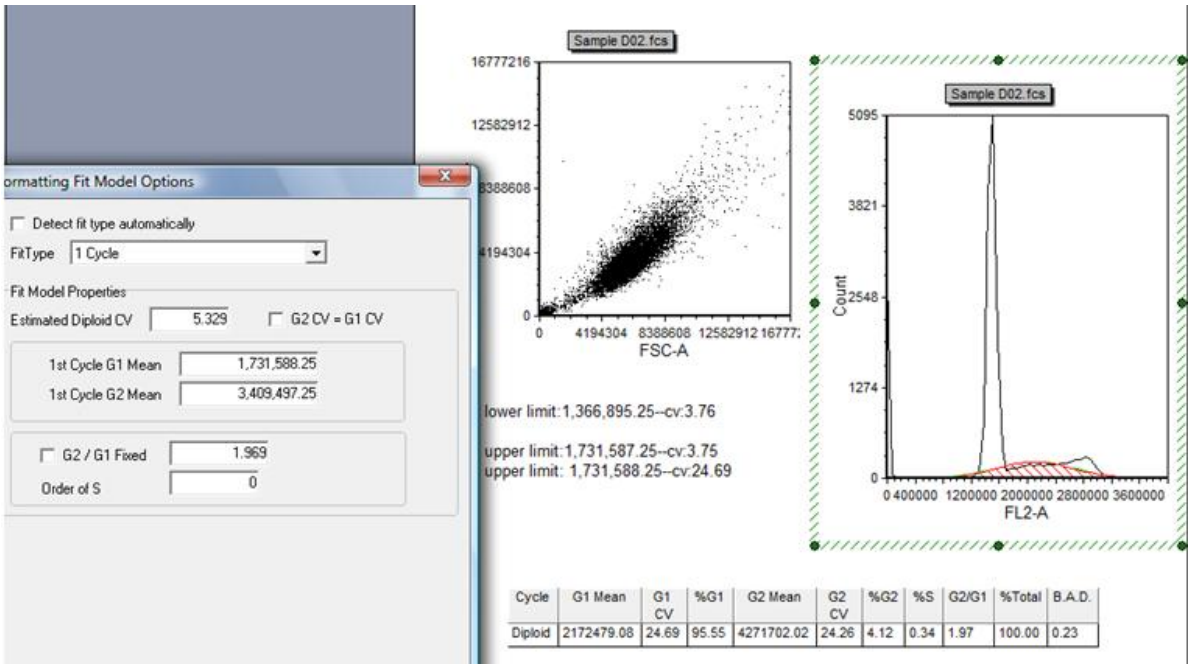


Figure H.3: *The dot plot, DNA histogram and its G1_CV values for lower and behind upper limits, and Formatting fit type Model Options window for sample D02 in experiment 23.*

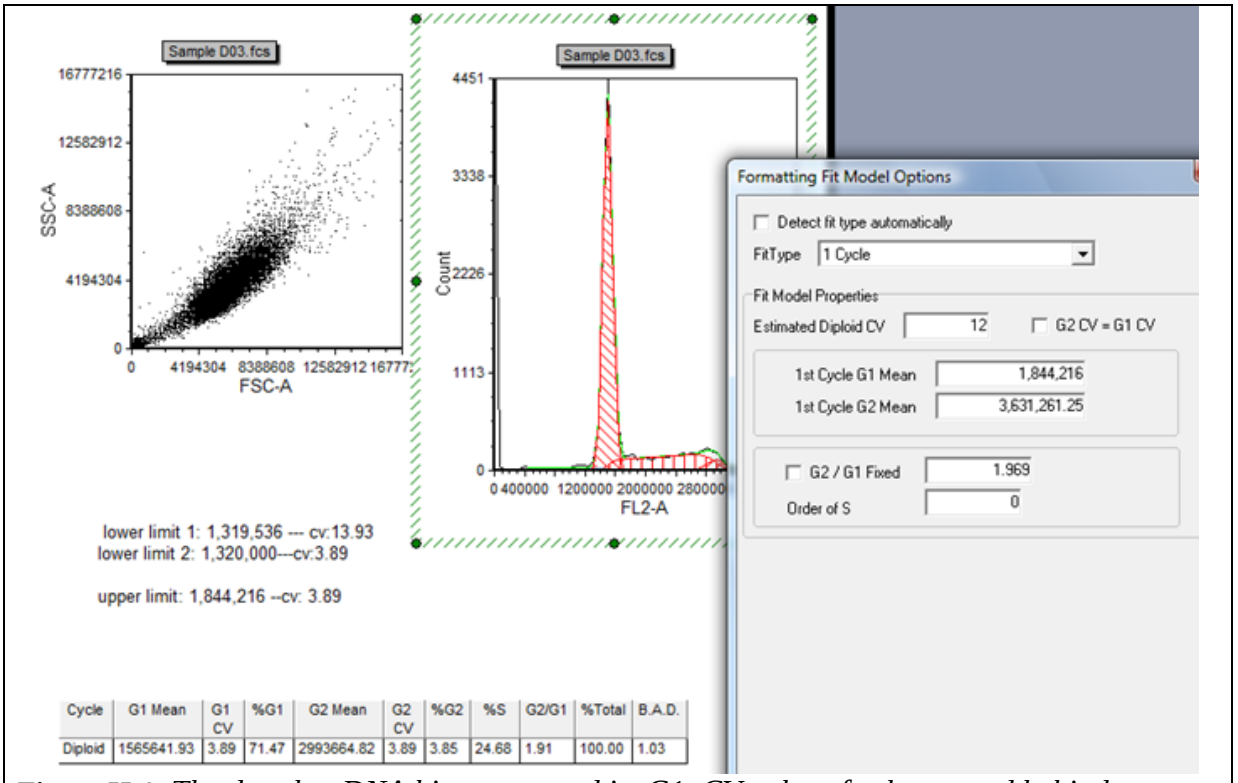


Figure H.4: *The dot plot, DNA histogram and its G1_CV values for lower and behind upper limits, and Formatting fit type Model Options window for sample D03 in experiment 23.*

Table H.1: The lower and upper channel numbers and their corresponding G1_CV values for three samples.

Sample	Channel number for lower limit for G1	Channel number for upper limit for G1	Lower G1_CV	Upper G1_CV	Difference between G1_CVs	The channel number difference
DO1	1,368,536	1,734,136	3.62	3.62	0.00	365,600
DO2	1,366,895.25	1,731,587.25	3.76	3.75	0.01	364,692
DO3	1,319,536	1,844,216	3.89	3.89	0.00	524,680

According to these results, for the reliability of the data ±0.01 *software error* has been added to G1_CV values in addition to the standard error.

Appendix I: The Acronyms

Bq	*Becquerel*
CB	*Citrate Buffer*
Cdk	*Cyclin – dependent –kinase*
Ci	*Curie*
^{60}Co	*Cobalt 60*
CRBC	*Chicken Red Blood Cells*
CSDA	*Continuous Slowing-Down Approximation*
CV	*Coefficient of Variation*
DNA	*Deoxyribonucleic acid*
DSB	*Double –Strand Break*
FCM	*Flowcytometric Measurement*
FITC	*Flourescein* isothiocyanate
FSC-A	*Forward Scattering- Area*
FSC-H	*Forward Scattering- Height*
Gy	*Gray*
HCC	*Hepatocellular Carcinoma*
HRR	*Homologous Recombination Repair*
HRS	*(Low Dose) Hyper Radiosensitivity*
ICCM	*Irradiated-Cell Conditioned Medium*
ICP	*Imspulscytophotometer*
IR-Model	*Induced Repair-Model*
IRR	*Increased Radio resistance*
LAF	*Laminar Flow*
LASER	*Light Amplification by Stimulated Emission of Radiation*
LDR	*Low Dose Rate*
LDRres	*Low Dose Rate Resistant*
LED	*Light Emitting Diode*
LET	*Linear Energy Transfer*
LQ	*Linear Quadratic*
NHEJ	*Nonhomologous End – Joining*
OER	*Oxygen Enhancement Ratio*
$OH^{\bullet}$	*Hydroxyl Radical*
PBS	*Phosphate Buffered Saline*
PI	*Propidiumiodide*
PMT	*Photomultiplier*
Rb	*Retinoblastoma Protein*
R-PE	*R-Phycoerythrin*
RPMI	*Roswell Park Memorial Institute*
SDS	*Sodium dodecyl sulphate*
SOBR	*Sum-Of-Broadened-Rectangles*
SSC-A	*Side Scattering- Area*
SSC-H	*Side Scattering- Height*