EFFECTS OF B-HYDROXYBUTYRATE IN COLON CANCER CELL METABOLISM

Science Research Project

EFFECTS OF B-HYDROXYBUTYRATE IN COLON CANCER CELL METABOLISM

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“What is a scientist after all?
It is a curious man looking through a keyhole,
the keyhole of nature, trying to know what’s going on.”
By Jacques-Yves Cousteau

Abstract

Do you know someone who had suffered cancer? Which type? Have you ever thought about the complexity of an illness, which even being highly studied in research groups around the world, is still killing millions of people per year? Have you ever heard about bioinformatics and its new approach in research? If you have heard or considered at least one of these topics, or you are simply interested in them, let me guide you through the complexity of our units of life, cells; its regulation, metabolism; the characteristics of cancer and the usefulness of computer experimentation, using the meticulous and rigorous scientific method applied to the following question:
"Does β-hydroxybutyrate affect colon cancer Metabolism?"

Resumen

¿Conoces a alguien que haya sufrido cáncer? ¿De qué tipo fue? ¿Alguna vez te has planteado la complejidad que puede tener una enfermedad, altamente estudiada por científicos alrededor del mundo y, aun así, seguir matando millones cada año? ¿Has oído hablar alguna vez de la bioinformática y su nuevo enfoque en investigación? Si has oído hablar de alguno de estos temas, o simplemente estás interesado en ellos, permíteme guiarte a través de la complejidad de la unidad de vida, la célula; su regulación, el metabolismo; las características del cáncer y la utilidad de la experimentación computacional, utilizando el meticuloso y riguroso método científico aplicado a la siguiente pregunta:
"¿Afecta el β-hidroxibutirato al metabolismo del cáncer de colon?"

Resum

Coneixes algú que hagi sofert càncer? De quin tipus fou? T'has plantejat mai la complexitat que pot tenir una malaltia que tot i ser altament estudiada per científics d'arreu del món segueixi matant a milions cada any? Has sentit mai a parlar de bioinformàtica i el seu nou enfocament a la recerca? Si n'has sentit a parlar o t'has plantejat algun d'aquests temes, o simplement t'interessen, permet-me guiar-te a través de la complexitat de la unitat de vida, la cèl·lula; la seva regulació, el metabolisme; les característiques del càncer i la utilitat de la experimentació computacional, utilitzant el meticulós i rigorós mètode científic aplicant-lo a la pregunta:
"Afecta el β-hidroxibutirat al metabolisme del càncer de colon?"

Acknowledgements

First of all, I would like to thank MyTutor, my tutor at the high school, and the PhD student that guide me along this process, for their patience, support, expert advice and huge inspiration. They were always helpful at the best moments and the ones I thought I would not be able to finish it, pressuring me, but also letting me autonomy to try a project, I would even say more, an adventure as amazing and educational as this project.

I would also like to thank all the PhD student's partners that were always there for any doubt I could have solving it with a smile in their face and, specially, Prof. Marta Cascante for letting me do this project at their laboratories.

I am also grateful to Aurelie Billard, my extracurricular English teacher, for her interest in learning while correcting any mistake I could do throughout this project.

Last but not least, I would like to express my gratitude to my family and friends who supported me and advised me whenever I need it.

INDEX

1. INTRODUCTION

At the beginning, this project was only going to be about bioinformatics, a really interesting field unknown by many people, so I thought it would be nice to focus my project on that to show everyone how many research tools there are on the internet and the awesome things you can achieve with them. Although, last year I was honoured to participate in the educational program *Crazy about Biochemistry* at the University of Barcelona (UB).

This was an inflection point in my research project, because the program let you develop your high school research project at their labs, so I could try something further than what I had expected. There were many things that could have been done, however I encountered with a PhD student, who is working in the *Integrative Systems Biology, Metabolomics and Cancer* research group (leaded by Prof. Marta Cascante) in the Biochemistry and Molecular Biology department of the UB. He taught me about metabolism, metabolic models and I could not resist the idea of trying a project involving those subjects.

Months happened while we were discussing what I could do to accomplish my aim to learn about metabolism while working *in silico. So finally* "Effects of β-hydroxybutyrate in the medium of a Colon Cancer Cell Culture" was born as my project. A complex research project strongly based on three different subjects in biomedical research: Metabolism, Cancer and Bioinformatics. Topics overviewed throughout this document.

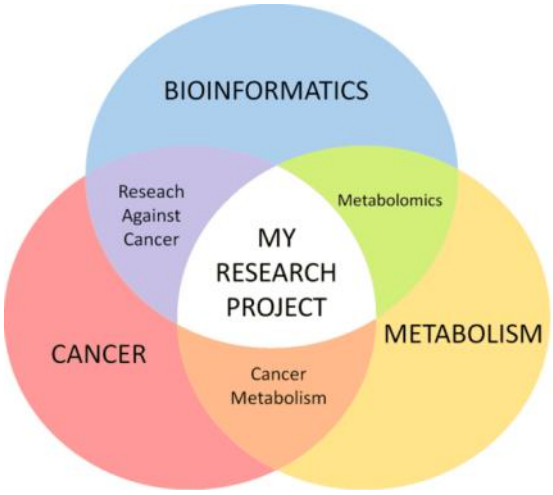


Illustration 1 is a Venn diagram that shows graphically how my project is interjected in these three subjects: A biochemical branch of study, metabolism; a severe illness, cancer; and an awesome tool, bioinformatics. As all of them have a role in my project they have a chapter each to explain the basis to learn and comprehend more about these amazing subjects.

1.1. MOTIVATION

At first, I just approached to this project as a way to see if I liked or not bioinformatics as I was considering studying it in the future. But as the biochemistry course was passing by I learnt a thousand new interesting fields and scientific concepts I would have never guessed they existed. As I am so curious, or at least I consider myself one, I could not let the opportunity of a professional research experience pass without taking the most of it, so I worked hard to find a way to do a project in which I could accomplish my objectives, but at the same time, I could learn a lot from different topics like metabolism, cancer...

1.2. OBJECTIVES

As I mentioned, my first two goals were to show an unknown tool like bioinformatics while understanding if that is what I want to dedicate my life on. But as everything evolved I started adding to the previous ones many things that at the end I could sum up as:

- Learning in depth about the three topics related to my project.
- Experience a complex research project with the most detail possible.
- Confirm or reject the hypothesis proposed at the Experimental part.

1.3. METHODOLOGY

This has been the hugest project I have ever been involved in, it has a huge biomedical and metabolomical aspect. Even so the tools and processes used are completely different from one another.

That is because nowadays experimentation requires a lot of technology from different fields so in order to carry out most of this project (the *in vitro* part) I've done eukaryotic cell cultures in laminar flux hoods, observations with an optic microscope and pictures with a computer attached, a cell counting analysis with

a tool called Scepter, Spectrophotometry with an automatic spectrophotometer[1], data analysis with Excel and statistic tests with R Studio.[2]

Finally, I also used Geany and Optflux to do the *in silico* experimentation.

1.4. TROUBLES

This project is definitely the hardest and longest project I have ever worked on, and as always the path to achieve your goals is full of hinders ready to test your skills to avoid or to get over them.

The main hinder when working on this project is the complexity of the topics that no one had never taught me with enough time, what supposed me many hours reading and finding information complex enough to show the reality of the bases underneath this project, but simple enough to be able to leave the main ideas clear.

Apart from that I have encountered other troubles like many computer crashes in both theoretic and practice parts; time and access limitations, because I had a few time to spent a t the UB to learn everything I would have need to do everything fluently and many articles and books were not at my reach so I had to ask to thirds to get them for me or just leave them apart.

Even all this I consider I have surpassed them correctly as this project is finally born.

[1] Scepter cell counting and Spectrophotometry are explained in the annex II page X.
[2] Excel calculation sheets and the R Script are in the annexes VII and VIII, respectively.

2. BASIC BIOLOGY AND CHEMISTRY

2.1. CHEMISTRY CONCEPTS

Metabolism is a biological and chemical complex process that involves a lot of different mechanisms for which it is necessary to revise some concepts from chemical reactions, biomolecules, cell structure... So we will start from the most basic concepts in Biology and Chemistry so we revise them all step by step.

Chemistry is defined by the Oxford Dictionary as *the branch of science concerned with the substances of which matter is composed, the investigation of their properties and reactions, and the use of such reactions to form new substances.* So what is it exactly matter?

We consider **matter** anything that has a mass, an amount of it is an object and it occupies a volume. The matter is made of atoms.

In this picture we can see a Helium **atom** and it is formed by electrons (grey) and a nucleus made of protons (red) andn (blue).

The **nucleus** is always at the centre of the atom and it is where nearly 100% of the mass is kept.

The **neutrons** are the subatomic particles with more mass, they have neutral charge and they may decay forming what we call isotopes (same number of protons but different number of neutrons).

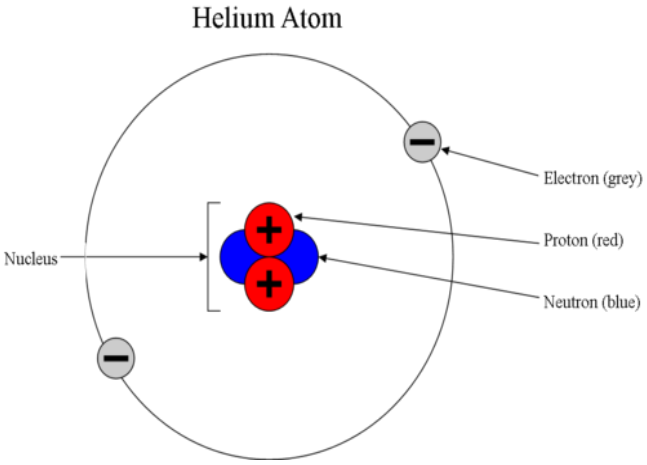


Illustration 2this picture is a representation of a Helium atom in the Bohrs atomic model with the name and the charge of each subatomic particles: Electrons negatively-charged (grey), Protons positively-charged (red) and Neutrons neutrally-charged (blue).

Protons mass much more than electrons, they have positive charge that keeps the electrons together and as it determines which element it is if they decay (due to radioactivity) the

element changes, it has different properties.

Electrons have a negligible mass, negative charge and if there are more than protons in an atom we call it a negative ion (anion) and if there are less than protons it is a positive ion (cation).

All the 118 elements are organized in the **periodic table** from 1 to 118 protons in their nucleus.

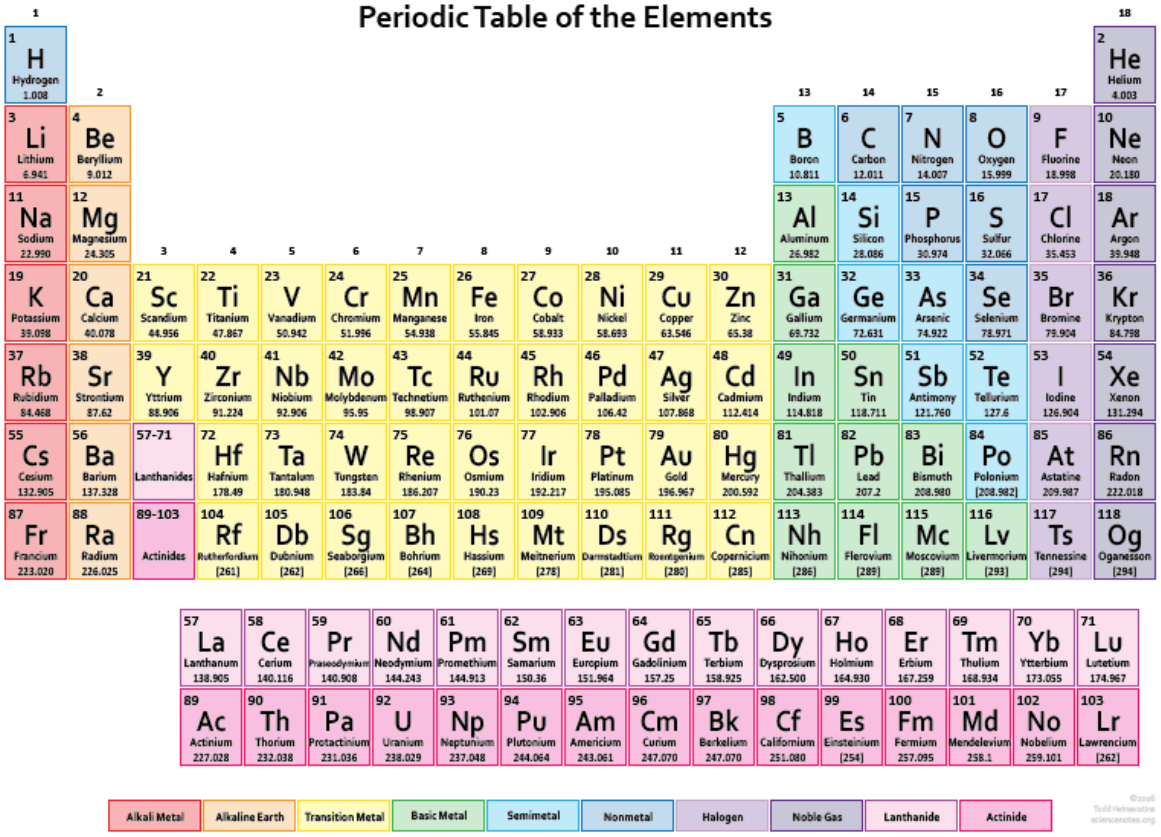


Illustration 3 This is the modern version of the periodic table with all the elements discovered until 2017. Although here there is an exhaustive classification and we will simplify it as metals (red, orange,yellow, green and the two pink rows), non-metals (light and dark blue) and Noble gases (the darkest purple, the group 18). Depending on the row and the group, they have different properties but as they are not strictly necessary the reasons why it has this shape won't be explained.

The elements in the periodic table sometimes bond with different elements or with atoms of their same element, when we have at least two atoms bonded we call them a molecule. Those **chemical bonds** can be classified in three different bonds:

Ionic bond: is formed by a metal and a nonmetal and in order to form it they

give and receive electrons so they attract as magnets.

Covalent bond: is formed by two nonmetals and their goal is to be in the distance where sharing their electrons with each other suppose the least amount of energy.

Metallic bond: is formed by two metals and they group their nucleus and let their electrons go everywhere around them.

2.2. BIOCHEMISTRY

Now we know basic concepts about molecules, what about the elements and molecules that form all the organisms, are they all those 118? No, a few elements form organisms but in really different arrangements. Those elements are called **Bioelements** or Biogenic elements.

Table 1 This table is an arrangement of the different elements of the periodic table that makes us all. From the main ones to those that are only a 0,0X% of our weight. The first two groups (Most important and Necessary) are compulsory in any organism; however the Variable ones are only necessary in some specific beings. 99% of organisms' weight is formed by the elements in the two first groups.

	Bioelements
Most important	Carbon (C), Hydrogen (H), Oxygen (O), Nitrogen (N), phosphorous (P) and Sulphur (S)
Necessary	Calcium (Ca), Sodium (Na), Potassium (K), Magnesium (Mg), Chlorine (Cl), Iron (Fe) and Iodine (I)
Variable	Bromine (Br) Zinc (Zn) and Titanium (Ti)

As molecules are arrangements of elements, bioelements bond to form **biomolecules**.

Table 2 Biomolecules classification.

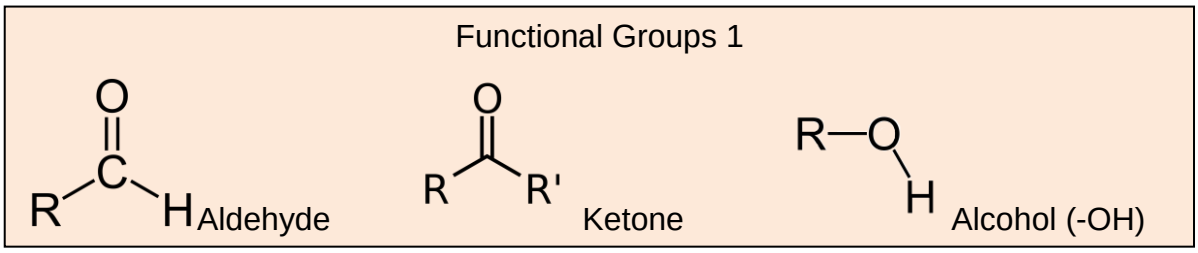
Biomolecules		
Simple	Compounds	
	Inorganic	Organic
Molecular oxygen (O_2) Molecular nitrogen (N_2)	Water (H_2O) Carbon dioxide (CO_2) Salts (NaCl, $CaCO_3$…)	Carbohydrates Lipids Proteins Nucleic acids

We will focus on the organic compounds as they are much more complex.

2.2.1. Carbohydrates

Carbohydrates are biomolecules made of C, H and O. Approximately twice H as much C or O ($C_nH_{2n}O_n$).

Its monomers are called monosaccharide. And those bond to produce disaccharides and polysaccharides. Shorter chains of this monomer are commonly known for their sweet taste.



2.2.1.1 *Monosaccharides*

They are made of between 3 to 7 carbon atoms bond with some hydroxyl and a ketone or an aldehyde functional group. Some monosaccharides, usually those with 5 and 6 carbon atoms are able to cycle.

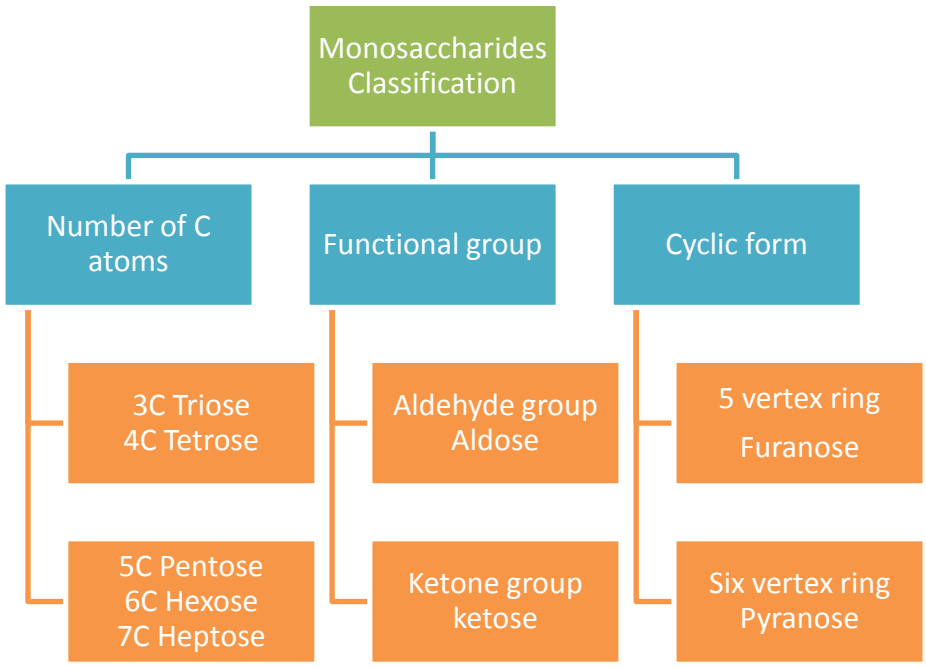
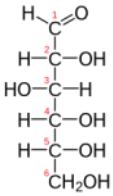
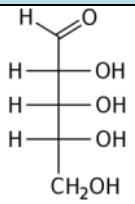
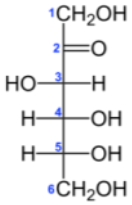
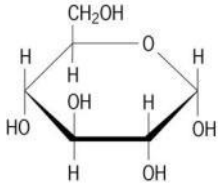
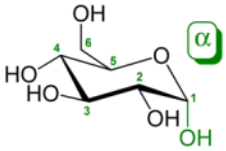
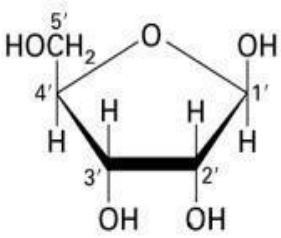
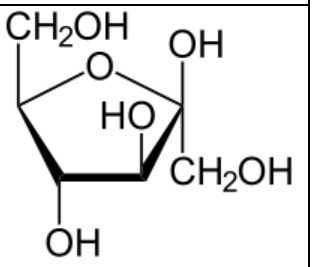


Diagram 1 In this diagram we can see three ways to classify the monosaccharides depending on: the number of carbon atoms, the functional group and in some cases their cyclic shape. However, there are some more involving the position of a hydroxyl group, optic activity, etc. Those are too technical for an outline.

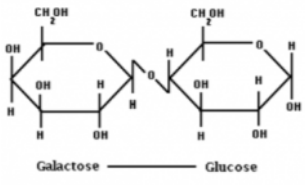
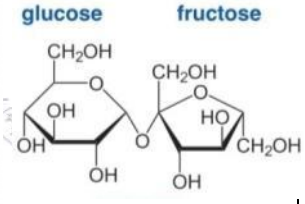
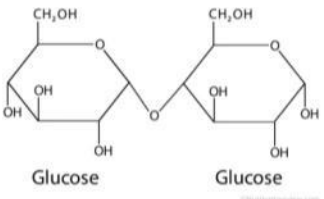
Table 3 Monosaccharide examples with their Fischer and Haworth projections which are able to switch from one to another and, also a classification based on the Diagram 1 concepts.

Monosaccharide	Glucose	Ribose	Fructose
Fischer Projection			
Haworth Projection	 It can also be seen like this: 		
Classification	Hexose Aldose Pyranose	Pentose Aldose Furanose	Hexose Ketose Furanose

2.2.1.2. *Disaccharides*

A disaccharide is a couple of monosaccharides bond together. If they both bond with the functional groups the molecule is no longer able to reduce any other molecule. The bond between two monosaccharides is called O-glycosidic bond or linkage.

Table 4 shows three different disaccharides and if they can reduce another molecule.

	Lactose	Sucrose	Maltose
Structure			
Reduction capacity	Yes	No	Yes

2.2.1.3. *Polysaccharides*

When many monosaccharides bond, they form a polymer called polysaccharide. Those can be made of just 1 kind on monosaccharide or made of two or more monosaccharides. Inside this group there are 4 really famous for their energetic or structural function:

-Starch and glycogen as energy storage in plants and animals, respectively.

-Cellulose and chitin as their main role in plant cell wall and insects' exoskeleton.

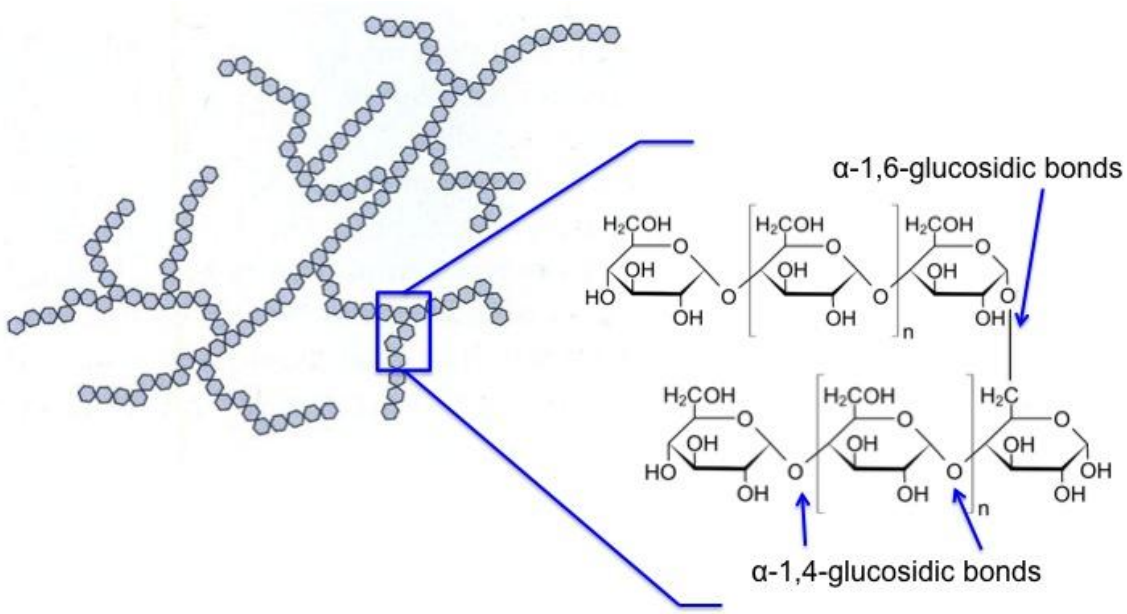


Illustration 4 This picture shows starch structure and bonds.

2.2.2. Lipids

They are the only organic biomolecules not formed by monomers, moreover they are pretty different from a structural point of view, but as they all share some key **properties** they are in the same group. Those properties are:

- Nearly insoluble in polar solvents
- Soluble in organic nonpolar solvents like alcohols, ethers, etc.
- Low density
- They leave a translucent mark on a paper.

Their functions are: energetic, reserve, structural, regulation, protection and thermal isolation.

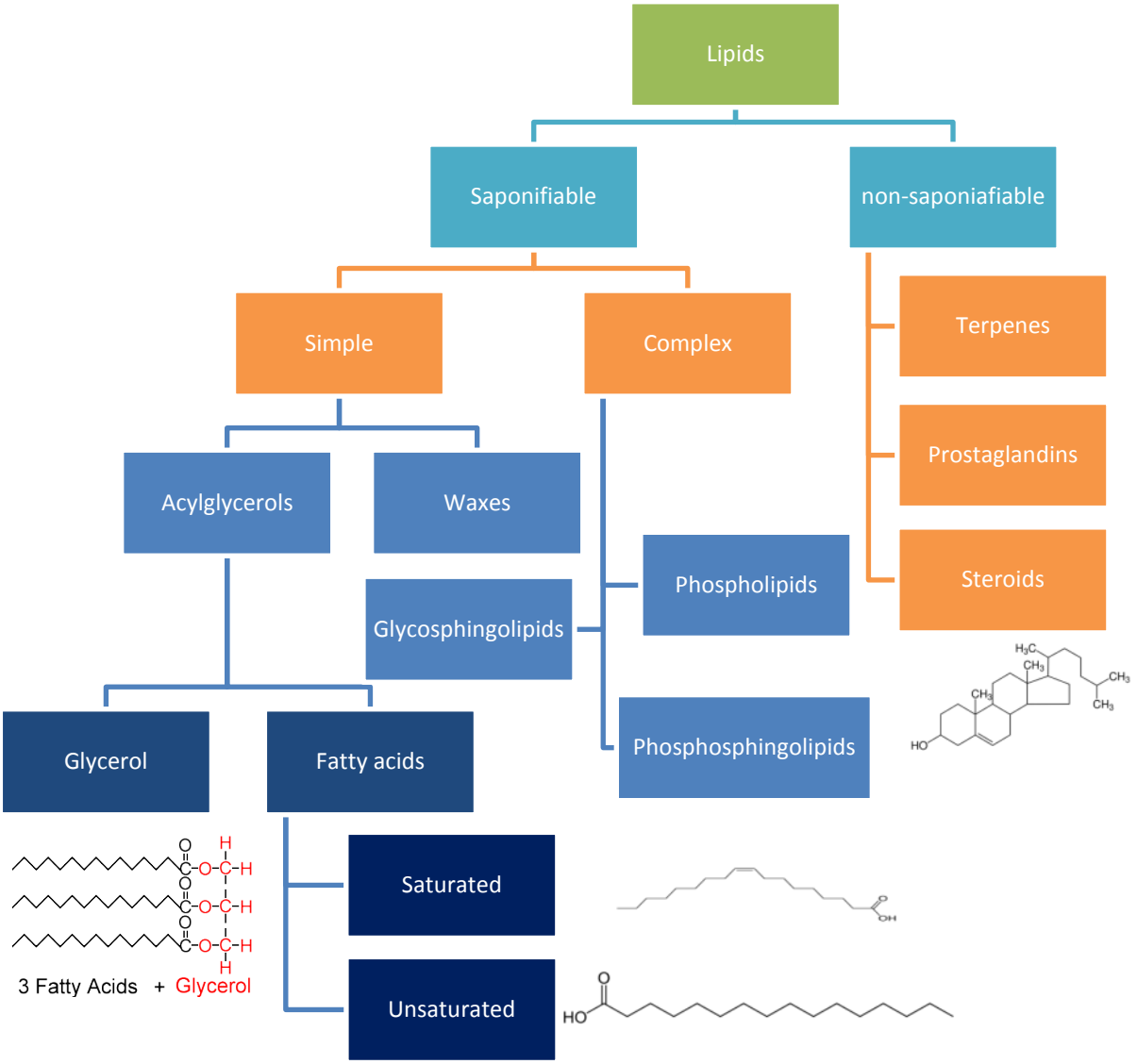


Diagram 2 Whole classification of lipids. Throughout the diagram we can see different ways to label them. In function of which reactions can be part of, if they are made up of just lipids or not, if they have double bonds along their structure, etc.

2.2.3. Proteins

Proteins are the most important biomolecules because they are the ones participating in each step of the long ensemble of processes we call metabolism. They are **polymers** of **amino acids**.

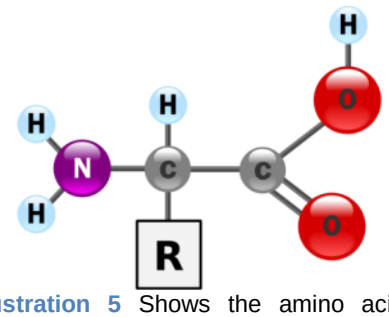


Illustration 5 Shows the amino acid structure. There is an amine group -NH2, a carboxylic acid group –COOH and a radical carbon chain (R).

An amino acid is a molecule formed by an acid and an amine functional group bond with a chain

that changes in each kind. There are 22 proteic amino acids, but there is one that is not encoded in our DNA, pyrrolysine.

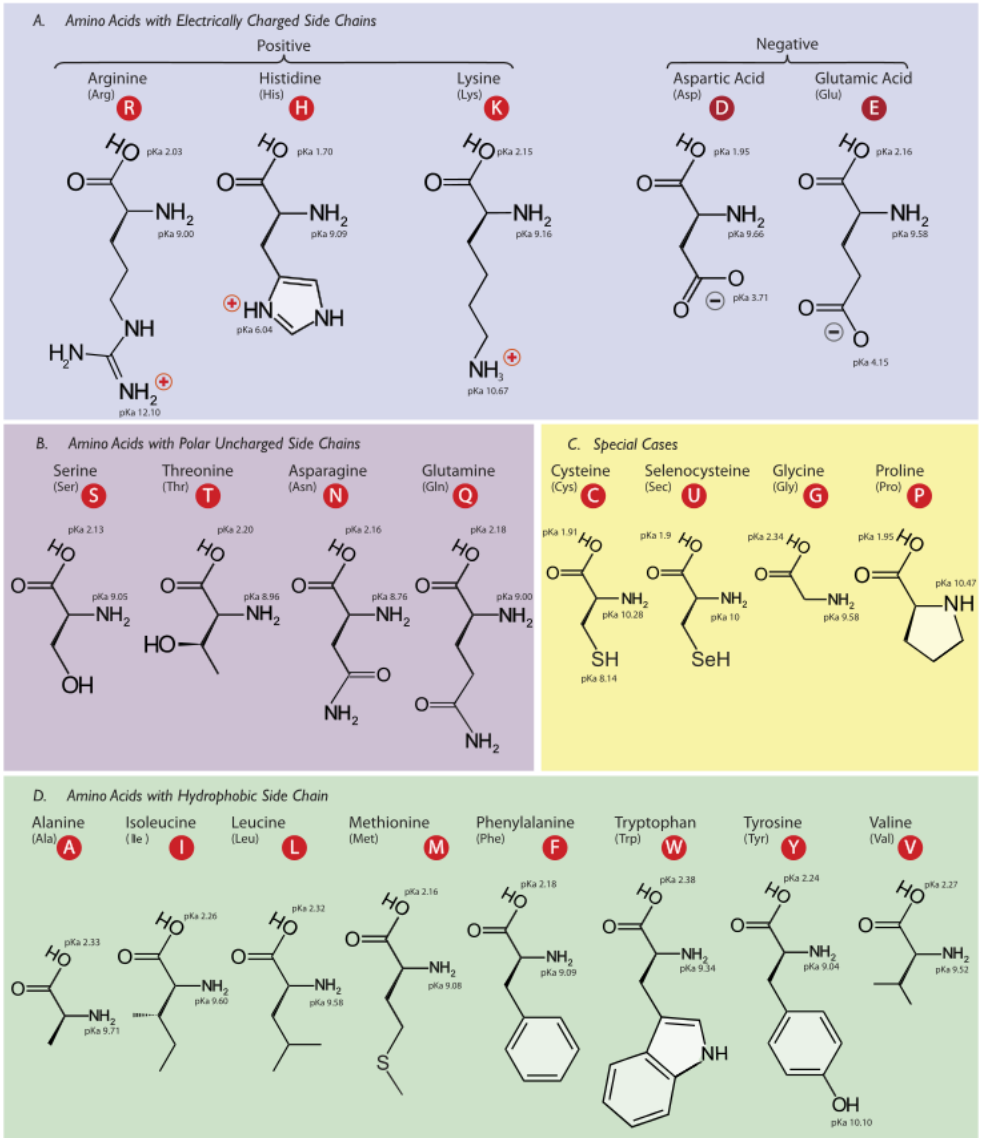
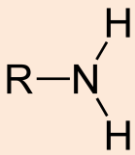


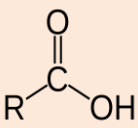
Illustration 6 This table show the 21 amino acids encoded in the human genome. This amino acids are sorted in function of their charge, if they are electrically charged, polar uncharged, nonpolar or hydrophobic and there are also separated those that have an special radical.

The amino acids bond their carboxylic acid group with an amine group from another in order to build a chain. The linkage between these two groups is called **peptide bond**. We consider any amino acid chain a peptide until this one is made of 100 amino acids.

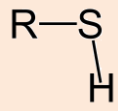
Functional Groups 2



Amine (Base group)



Carboxylic acid (acid group)



Thiol

Proteins have 4 structural levels, each one more complex than the one before

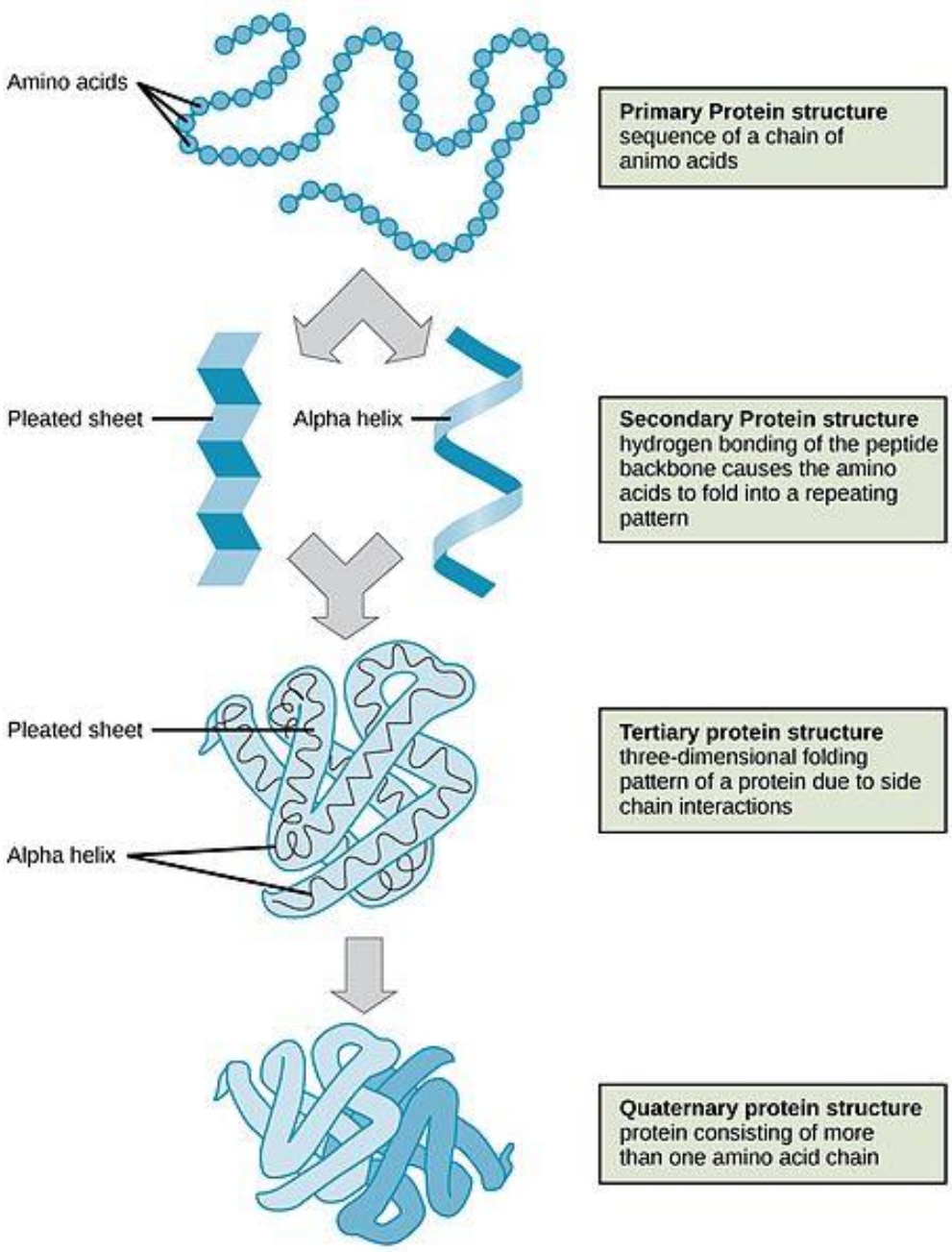


Illustration 7 This picture shows the four structures with a little explanation. This is the simplest scheme because there are more helixes than helix α like helix 3_{10} or helix π among other structures. Moreover, this model only applies for globular proteins as filamentous proteins do not fold the same way after secondary structure.

This structure developing process can go backwards, denaturalization. Higher structure bonds are sensitive to temperature, because most bonds are intermolecular (hydrogen bonds and Van der Waals interactions) which are not as strong as ionic or covalent bonds. Their charges can also change with the PH as each amino acid (so the protein) can work as a base or an acid

depending on the PH so their charge will change and with that their interactions. The acid-base behavior is called amphoterism.

Depending on the amino acids and the structure they end up the protein will have a different function. The main **functions** are:

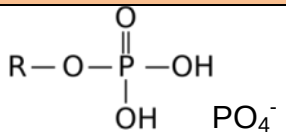
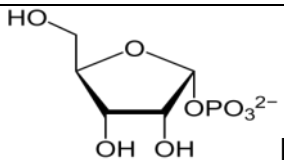
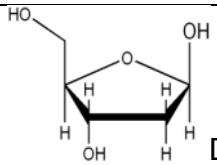
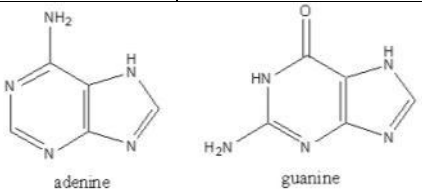
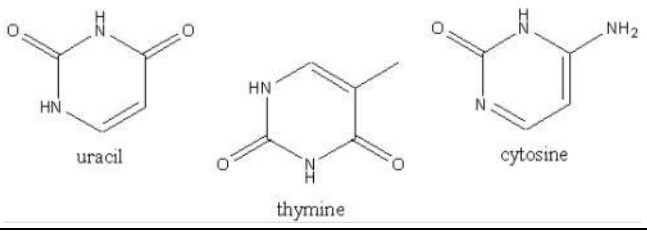
- Structural
- Transport
- Reserve
- Enzymatic
- Hormonal
- Defense
- Contractible
- Homeostatic

2.2.4. Nucleic acids

Nucleic acids are really important as they encode our **genetic information**, the proteins we will express, and others will keep the energetic and reductive potential used in the metabolism.

The basic monomers of nucleic acids are nucleosides and nucleotides. A nucleoside is a nitrogenous base bonded with a pentose, alike a nucleotide that is also a nitrogenous base bo.nded with a pentose although it is also bonded with a phosphate ion.

Table 5 Nucleic acid building blocks.

Nucleic acids molecules		
Phosphate		
Pentose	 Ribose	 Deoxyribose
Nitrogenous bases	Purines	 adenine guanine
	Pyrimidines	 uracil thymine cytosine

Depending on their structure and function we would distinguish two groups:

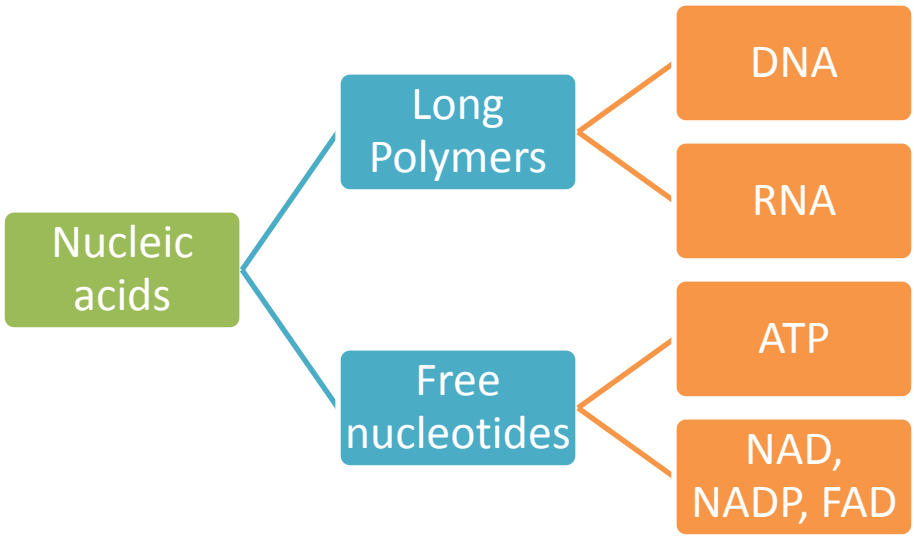


Diagram 3 In this diagram we divide nucleic acids in chains of nucleotides and free nucleotides, each group has completely different functions.

Adenosine triphosphate (**ATP**) is a nucleotide with 3 phosphate groups that keeps energy in its phosphate-phosphate bonds for enzymatic use.

Nicotineamide adenine dinucleotide (**NAD**) and its phosphate version (**NADP**) and Flavin adenine dinucleotide (**FAD**) are redox cofactors or, in other words, molecules that store or give electrons to change some molecules.

RNA is a one stranded chain of nucleotides with many different functions and shapes. Its nitrogenous bases are Adenine, Uracil, Cytosine and Guanine. The most famous RNAs are:

- mRNA: the molecule that carries the information from the DNA to the ribosome.
- tRNA: the RNA that brings the amino acids to the ribosome to produce proteins
- rRNA: half of the ribosomes' structure.

DNA is the most famous nuleic acid as it is the

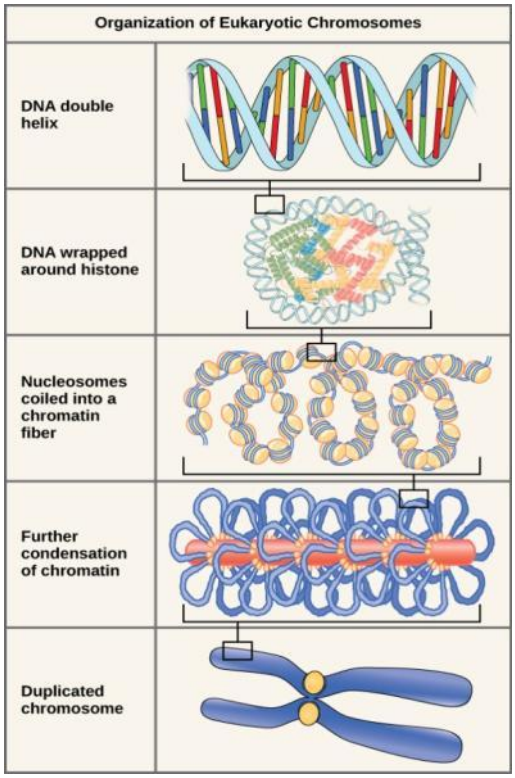


Illustration 8 is a representation of DNA Organization levels in Eukaryotic cells.

one that keeps our genetic information. It is stored in the nucleus (in eukaryotic cells) as an antiparallel double stranded chain of nucleotides. The nitrogenous bases that form it are Adenine, Thymine, Guanine and Cytosine.

2.3. CELL BIOLOGY

Cells are the minimum expression of life and there is nothing considered alive smaller than that. Talking about cells we must differentiate two groups: Prokaryotes and Eukaryotes. Prokaryotes do not have a differentiated nucleus, whereas **Eukaryotes** have it. We can also divide it in two more groups if they are from **animals** or plants. Plant cells have Chloroplasts and cell wall.

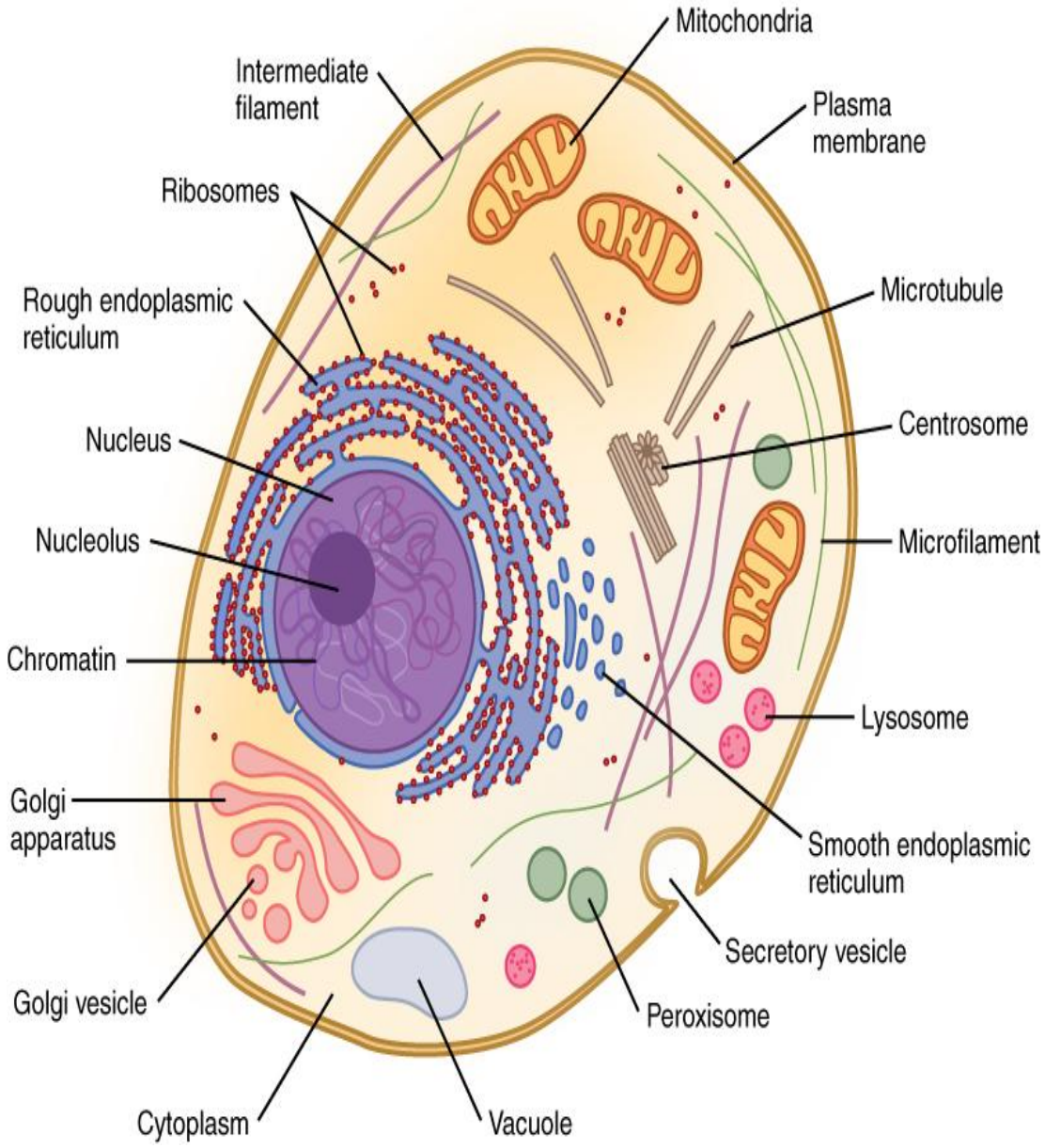


Illustration 9 Representation of an animal eukaryotic cell slice with its organelles named.

2.3.1. Organelles

1. **Plasma membrane**: It is the structure that demarcates the cell size and shape. It is made of proteins, phospholipids and carbohydrates.

-Phospholipids have polar and nonpolar edges they arrange with their polar head facing the liquid no matter if extracellular or cytosolic.

-Proteins that only face one side are peripheral proteins, and the ones that face both are transmembrane proteins. Proteins have many different functions that can be seen in proteins part.

-Carbohydrates are used as cellular markers, they are attached to lipids and proteins forming the glycocalix a structure used by the immunologic system to recognize the cells.

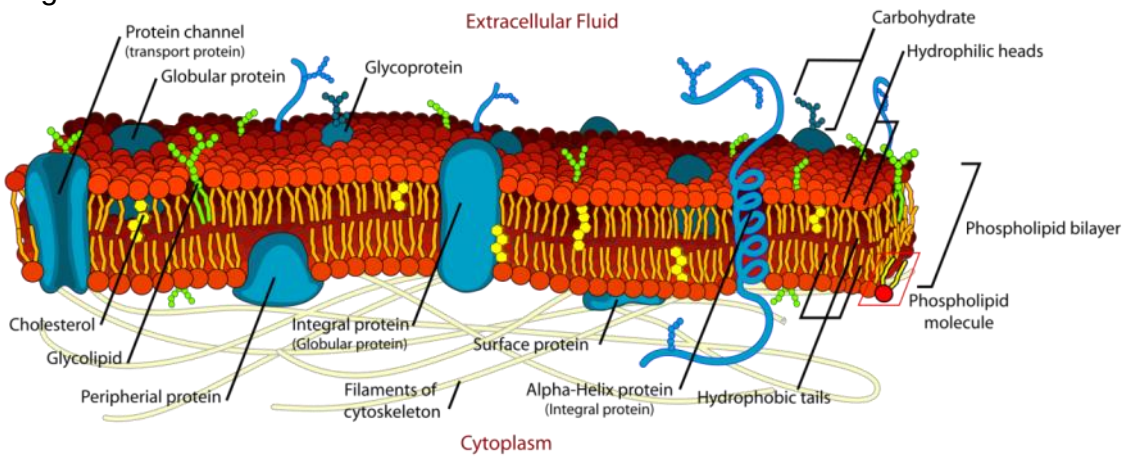


Illustration 10 is a picture of the plasma membrane and the different molecules that form it.

Secretion membranes: Are vesicles formed on the cell membrane to let big nutrients enter the cell and excrete cellular wastes. There are different secretory vesicles, depending on the function.

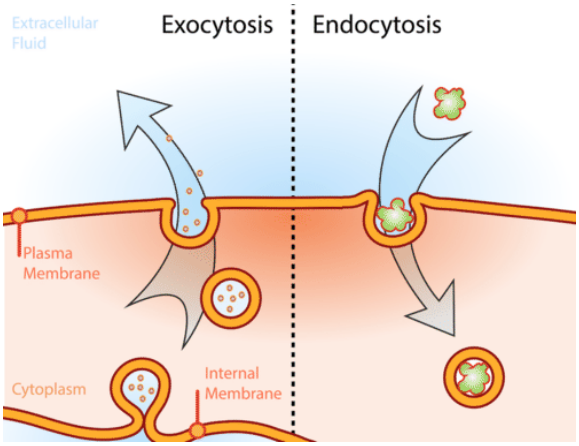


Illustration 11 This picture shows two different secretory vesicles:
On the right, one vesicle is doing the exocytosis, a process in which the cell secretes substances (usually wastes).
On the right, instead, it is doing the endocytosis, a process in which the cell collects huge molecules from the extracellular medium.

2. **Cytoplasm**: is the intracellular space, it contains the cytosol, the cytoskeleton and many organelles.

-Cytosol is the name that receives the liquid medium inside the cell. It is mainly water and salts.

-Cytoskeleton is a structure made of different string of proteins that keeps and organizes all structures in their respective places, gives certain mobility and, like in muscular tissue, contracts.

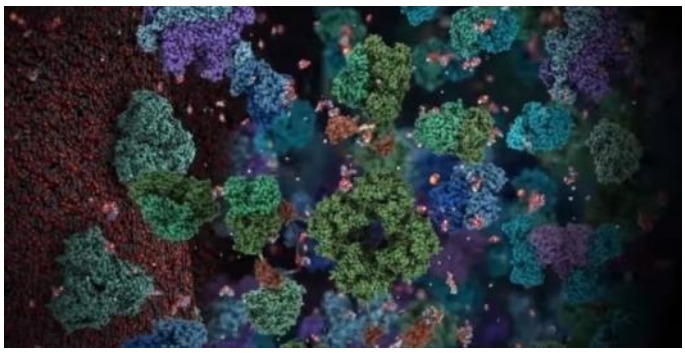


Illustration 12 This picture shows a computer representation of the cytosol, a place full of different proteic structures like enzymes, cytoskeletal fibres and many more structures and molecules that constantly affect each other.

There are three different cytoskelletal fibers:

- Microtubules
- Intermediate filament
- Microfilaments

3. **Centrosome**: is the organelle that rules microtubules movement.

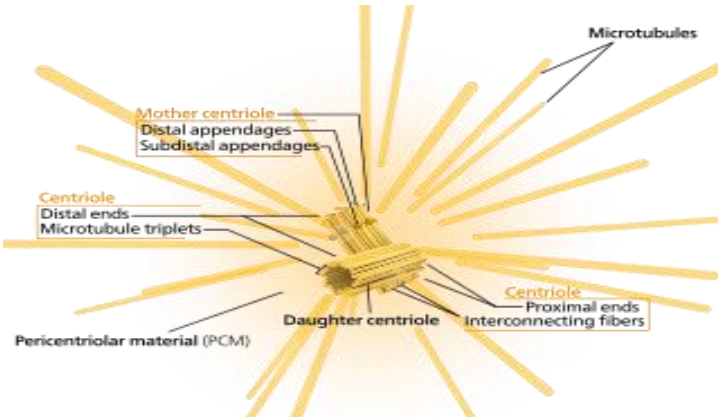


Illustration 13 As we can see in the picture the centrosome is made of two centrioles with a complex structure that enables the management of most cellular microtubules.

4. **Ribosomes**: Are the organelles that synthesize the proteins from the mRNA. Those are made of rRNA and proteins. They are located in the cytosol or in the walls of the endoplasmatic reticulum. They are 50% proteins and 50% rRNA.

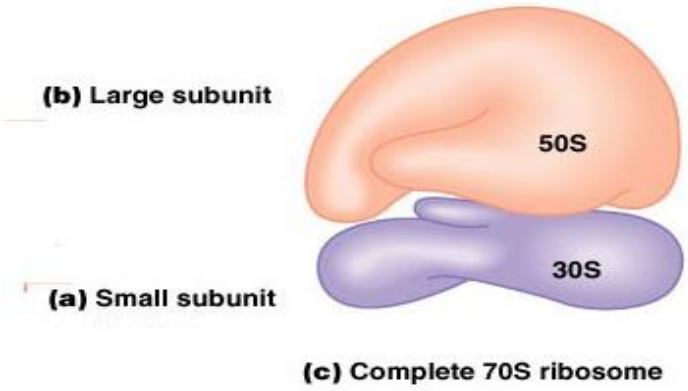
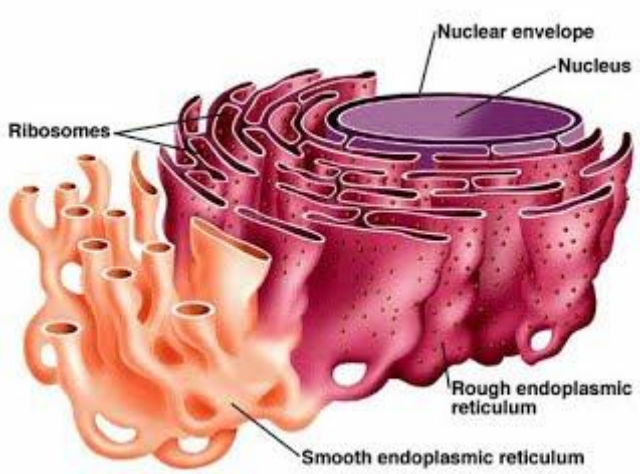


Illustration 14 As we can see in the illustration, ribosomes are made of two subunits, one is larger (50S) than the other (30S), and although their composition is really similar, their structure and function during the translation is really different.

5. **Endoplasmatic reticulum** (ER): There are two types: rough and smooth. Each type has a different function and structure, but they are both attached together to the nucleus envelope. The functions may vary like detoxification, plasma membrane production (proteins and lipids), etc.

Illustration 15 is a picture of the ER attached to the nuclear envelope. It shows the different parts it is divided into.



6. **Golgi** apparatus: is the organelle that produces vesicles and completes proteins and lipids synthesized in the ER and capsules them in vesicles to attach to the plasma membrane.

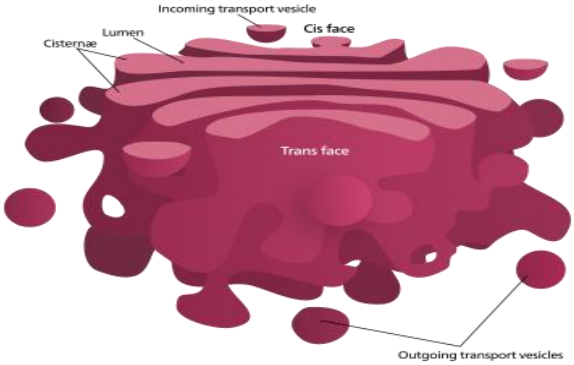


Illustration 16 This organelle is formed by vesicles and up to 30 cisternea. It has two differentiated faces: Trans and Cis. The name of the face depends on what is facing the ER or the plasma membrane.

The vesicles produced in the Golgi apparatus can be: lysosomes, peroxisomes

and reserve vacuoles.

Lysosomes and peroxisomes contain enzymes used to digest certain molecules that could be harmful outside these membrane vesicles.

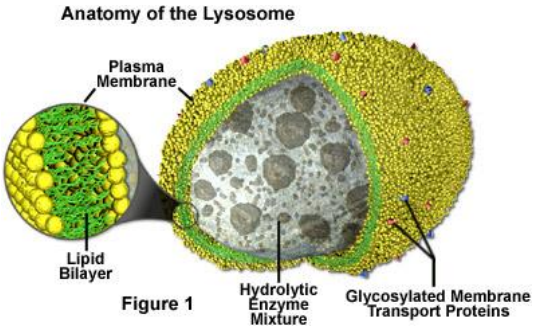


Illustration 17 is a computer representation of a lysosome and its parts: enzymes and a lipid bilayer.

Vacuoles mostly have a storing function, so they keep different substances like water, proteins, etc. inside their membrane.

7. **Mitochondria**: is basically the powerhouse of the cell, it is the organelle where the aerobic metabolism is done and it is the metabolic pathway that most chemical energy produces in human cells.

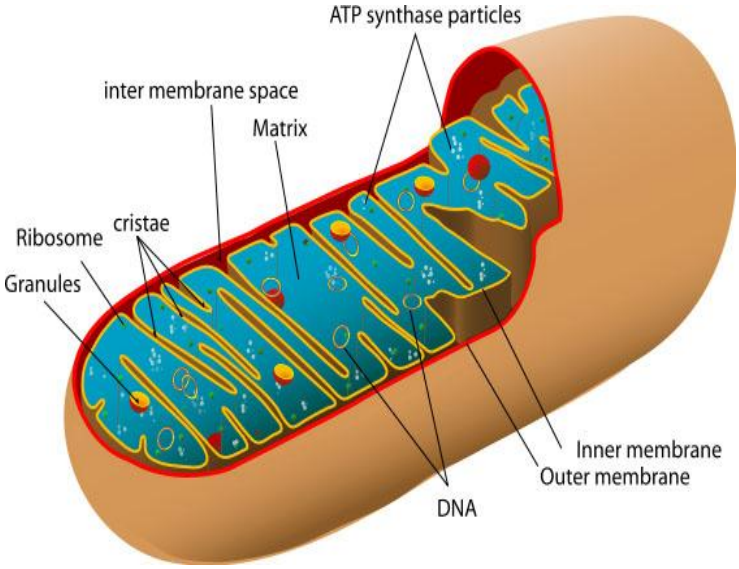


Illustration 18 is a simplified representation of the mitochondria. Mitochondria is made of two membranes, the outer and round and the inner one shaping cristae. Inside this second membrane there are cyclic DNA strands, Ribosomes, lots of enzymes. Mitochondrias are supposed to come from the association of two primitive cells. This would explain why this organelle has similarities with bacteria like cyclic DNA.

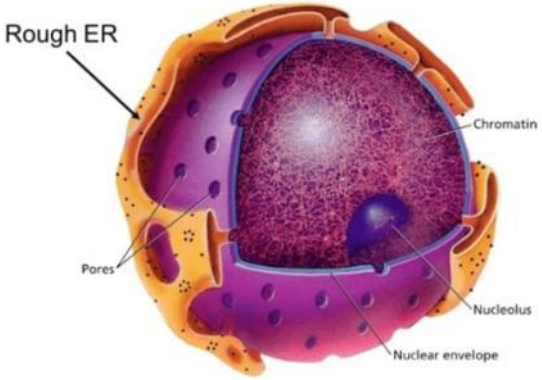
8. **Nucleus**: is a porous spherical structure where DNA is stored, replicated and transcribed. Inside the nucleus are produced mRNA, iRNA, miRNA, hnRNA,

rRNA and many more.

-Nucleolus is a nRNA structure where rRNA is synthesized.

-DNA: is the genetic material of the organism, and it can be in two different shapes: one string-like called Chromatin and one.

Illustration 19 shows the nucleus and its parts.



2.4. CHEMICAL REACTIONS

First of all in order to understand what a chemical reaction is we must understand when a change is physical or chemical.

A **physical change** is the one the matter composition doesn't change at all; instead of a **chemical change** that is when matter changes its composition.

We say that the composition changes when the elements that form it change their organization so their bonds change.

A **chemical reaction** is the way matter changes. It is expressed by the **chemical equation** which shows the molecules that react (**reagents**) and the molecules that are produced (**products**). Between the reagents and the products there is an **arrow** that shows the side of the reaction. The chemical equation also contains the proportion in which the reagents react; these are represented by numbers before the compounds (**stoichiometric coefficient**).

$$Reagents \rightarrow Products$$

$$C + O \rightarrow CO$$

$$Reagents\ or\ products \leftrightarrow Reagents\ or\ products$$

$$2\ NO_2 \leftrightarrow N_2 + 2\ O_2$$

Equations 1 These are two different types of chemical equations, the first ones are irreversible and the second ones are reversible. When a reaction is reversible, reagents and products react constantly if the minimum conditions are required (for example energy)

There are a lot of different reactions and we have many ways to classify them. We can take in account the nature of the reagents, the energetic, the kinematics, etc.

If we consider the nature of the reagents we find a very common reaction in which there is transference of electrons between the reagents, the **reduction oxidation reactions** or also known as Redox**.**

If we talk about the energetic, there are two different types of reactions:

- **Exothermic**, the energy of the products is less than the reagents so it is release as heat.
- **Endothermic**, the opposite than the last one so it collects energy from the environment.

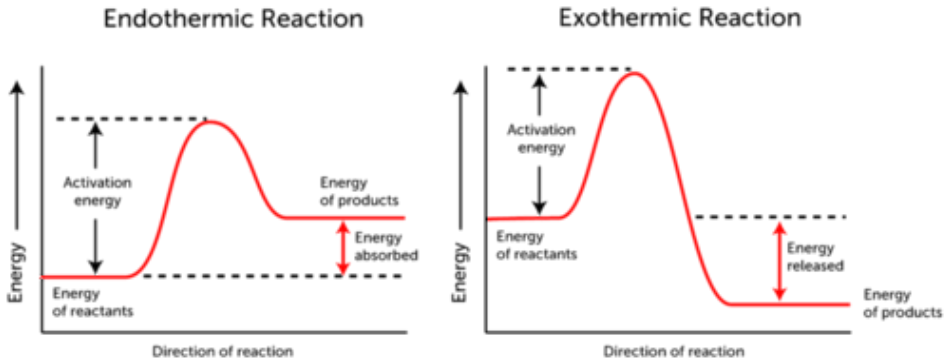


Illustration 20 is a graphical representation of the energy variance during an exothermic and an endothermic reaction.

Both types need an energy uptake at the first that is called **Activation energy**, which is retorned at the end so it does not affect the two types explained before, but that is requiered for the reaction to start functioning.

To start reactions easily and also speed them up there some chemical components that are called **Catalysts** that do this. Catalyst can be chemical or biological but both share two properties:

- Do not consuming during a reaction
- Initiating or accelerating the reaction.

3. METABOLISM

3.1. INTRODUCTION

Nowadays science has discovered things far away from our comprehension in fields like physics and its quantum mechanics that even being real particle descriptions, they are difficult to digest its random behaviour. But now try to explain what life is? Or consider at which point you can be considered alive or not. Maybe at first the answer seems easy but biologically and chemically it is not that easy. But what we already know is that life is sustained by metabolism.

Metabolism is defined as the amount of chemical reactions which happen inside the cells in order to produce biomass to grow (anabolism) and energy (catabolism) to develop the vital functions: nutrition, reproduction and relation.

3.2. ENZYMES

Enzymes are biocatalysts, so they have the two catalyst properties: participating but not consuming after a reaction and starting or accelerating reactions so they happen at the pace the cell needs the products.

Apart from the other catalysts enzymes have 4 more properties:

- Specificity: they only work with specific molecules.
- The temperature of the organism who synthesized them is the one they can work correctly.
- They are really active: they can increase the speed reaction up to 1 million times.
- They have a high molecular weight.

We can classify proteic enzymes as entirely protein made and those which need non-proteic part (holoenzymes), the proteic part is the apoenzyme and the other is the cofactor.

3.2.1. Cofactors

There are many different types of cofactors. These can be organic or inorganic.

Metallic ions like Calcium (Ca^{+2}) are inorganic ions and organic cofactors are also called coenzymes and those can be some really different molecules.

3.2.1.1. *Coenzymes*

Coenzymes are organic molecules that get attached to an apoenzyme to donate or receive chemic groups (atoms or little groups of atoms with a function). They are not usually specific of a single apoenzyme, there are many enzymes that use them.

These coenzymes can be divided in two groups:

- Redox coenzymes: These molecules transport protons (H^+) and electrons (e^-). The most important ones are the following which are based on nucleotides:
  - Nicotinamide adenine dinucleotide (NAD)
  - Nicotinamide adenine dinucleotide phosphate (NADP)
  - Flavin adenine dinucleotide (FAD)

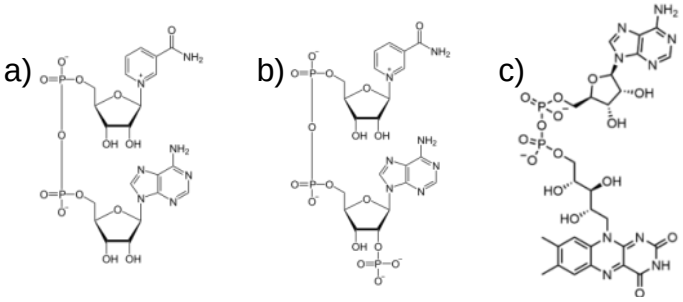


Illustration 21 is the three Redox enzymes. **a**) NAD **b**) NADP **c**) FAD

For example, the NAD^+ reaction is:

$$NAD^+ + 2H^+ + 2e^- \rightarrow NADH + H^+$$

- Transference coenzymes: These transport chemical radicals. The most important ones are:
  - Coenzyme A (CoA-SH). It uses its thiol group to do tioesters.
  - Adenosine phosphate family (ATP, ADP and AMP). They transport phosphate groups and the energy in their bonds.

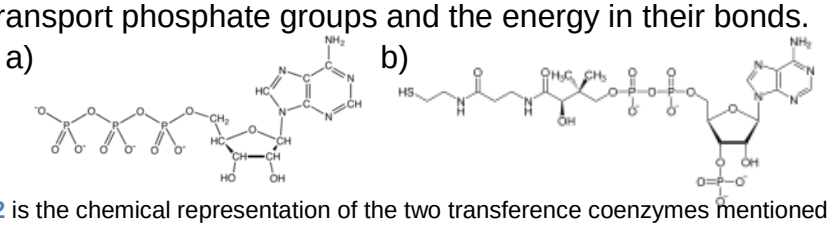


Illustration 22 is the chemical representation of the two transference coenzymes mentioned above. **a**) ATP **b**) Coenzyme A.

3.2.2. Enzymatic Reactions

In most chemical reactions the molecules that react are called reagents, but when an ezyme catalyzes it, we say it is an enzymatic reaction and instead of reagents, they are called substrate.

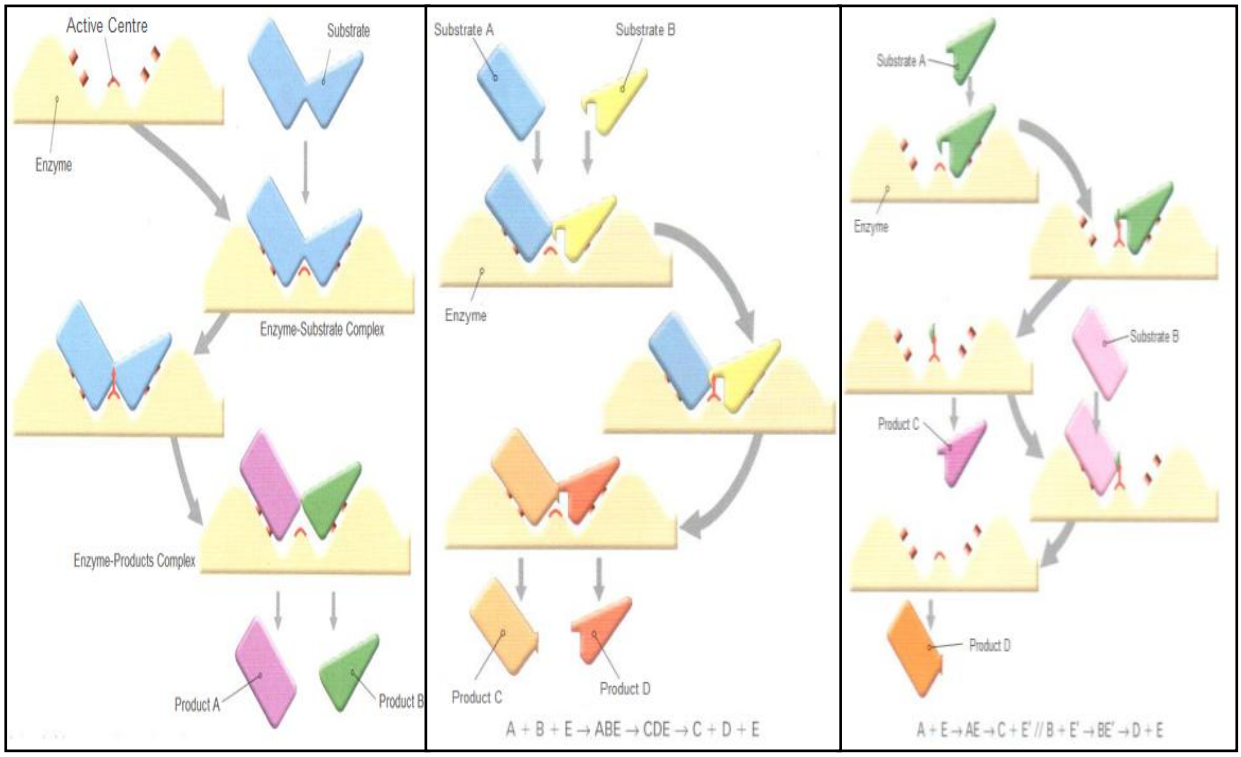


Illustration 23 represents three different ways enzymes can attach to substrate (these are a simplified and a schematic version). From left to right the picture shows the enzymatic activity with one substrate, with two substrates at the same time and with two successive substrates. There are thousands of enzymes and those attach with specific bonds so these are enough to appreciate how the substrate attaches to the enzyme forming a complex and then the reaction takes place, producing new molecules and then leaves them in the medium to repeat the reaction again with new substrate.

Enzymes attach to the substrate in the active centre, to do so, there are two known models:

- "Lock and key" model in which the enzyme attaches perfectly to the molecule.
- Induced fit model in which the enzyme adapts its shape to catch the substrate correctly.

3.2.2.1. *Inhibition*

Most enzymes are proteins so they are really sensible to temperature and pH values. Only some species have evolved to adapt their proteins to stand extreme condition, and humans are not one of those. Only a few degrees of

variation can denatulazise some amino acids in many enzymes. But our metabolism has evolved to have regulation systems like inhibition with specific molecules called inhibitors. This inhibition can be:

- Irreversible: when you can not undo the inhibition as the inhibitor has changed enzyme composition after attaching to the active centre.
- Reversible: when the enzyme is inhibited temporarily
  - Competitive: when the inhibitor "competes" with the substrate to enter the active centre.
  - Non competitive: when the inhibitor has a special point of attachment that unutilizes the enzyme whether there is substrate or not.

3.2.3. Types of enzymes

There is a classification for enzymes depending on their function.

1. Oxidoreductases: The enzyme participates in redox reactions.
   a) Oxidases: The enzyme oxidise substrate
   b) Dehydrogenases: separate H from the substrate.
2. Transferases: The enzyme transfer radicals from one substrate to another.
3. Hydrolases: The enzyme uses water to transfer H^+ and OH^- groups to the substrate.
4. Lyases: The enzyme adds or removes groups by removing or forming double bonds, respectively.
5. Isomerases: The enzyme changes the substrate to other isomeric form.
6. Ligases: the enzyme puts together the substrate with the energy of the ATP.

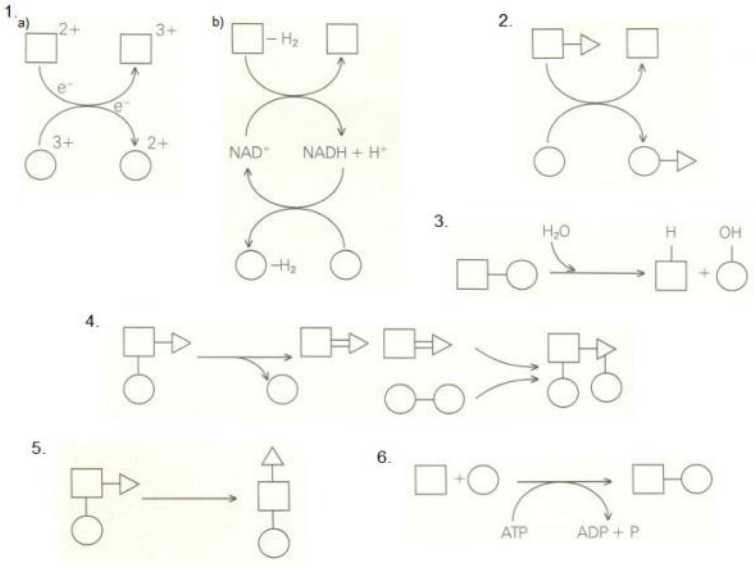


Illustration 24 Schematic reactions enzymes may do. The numbers are the same from the list next to it.

3.3. ROLE OF METABOLISM

As it is defined in the beginning of the metabolism chapter, metabolism has two goals, producing biomass and energy.

So we can differentiate metabolic reactions in two groups, in function of what their aim is:

- Producing energy.
- Producing structural or functional molecules.

Catabolism is the set of reactions which transform complex molecules into simpler ones obtaining energy. Anabolism, instead, is the synthesis of complex molecules using energy and monomers.

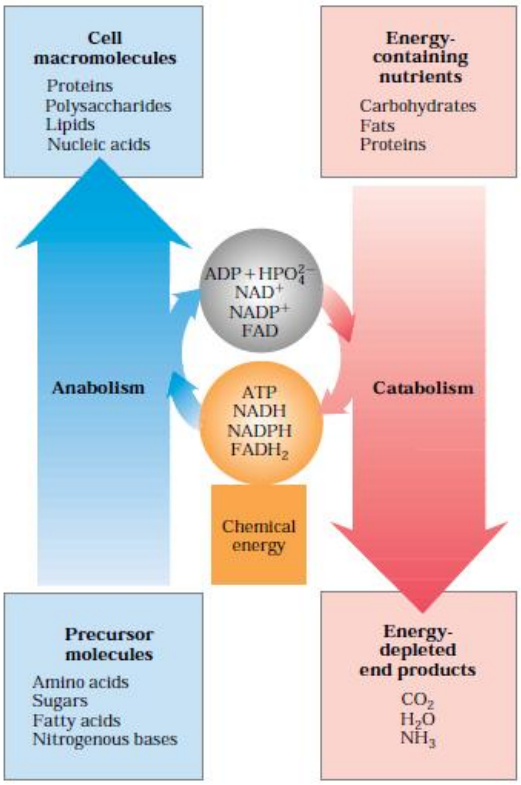


Illustration 25 This is a graphical representation of the molecules that are used and produced in Catabolism and Anabolism.

Nevertheless, as there are thousands of reactions, this classification is too vague, so we classify the reactions looking at what molecules they start with and which ones they produce in packs of a few reactions. Those packs are called metabolic pathways, and there are many, although nowadays we know it is not the best way to understand metabolism as it is too complex taking in account the quantity of metabolites and enzymes involved.

3.4. METABOLIC PATHWAYS

There are many different metabolic pathways which have really specific functions so the ones following the next constraints will be explained:

- Human metabolism, so it excludes a lot of different paths like the autotrophic anabolism.
- Main energy producer pathways.
- Pathways involved in cancer development and those taken in account during the experimentation.

3.4.1. Glycolysis

Carbohydrates are the most important source of energy in daily bases, as the brain mainly consumes glucose and the other two sources of energy (lipids and proteins) are only used in absence of the previous one.

The pathway that starts glucose catabolism is Glycolysis. Although this pathway can start with other monosaccharides they must be changed to any of the carbohydrates of this pathway before entering.

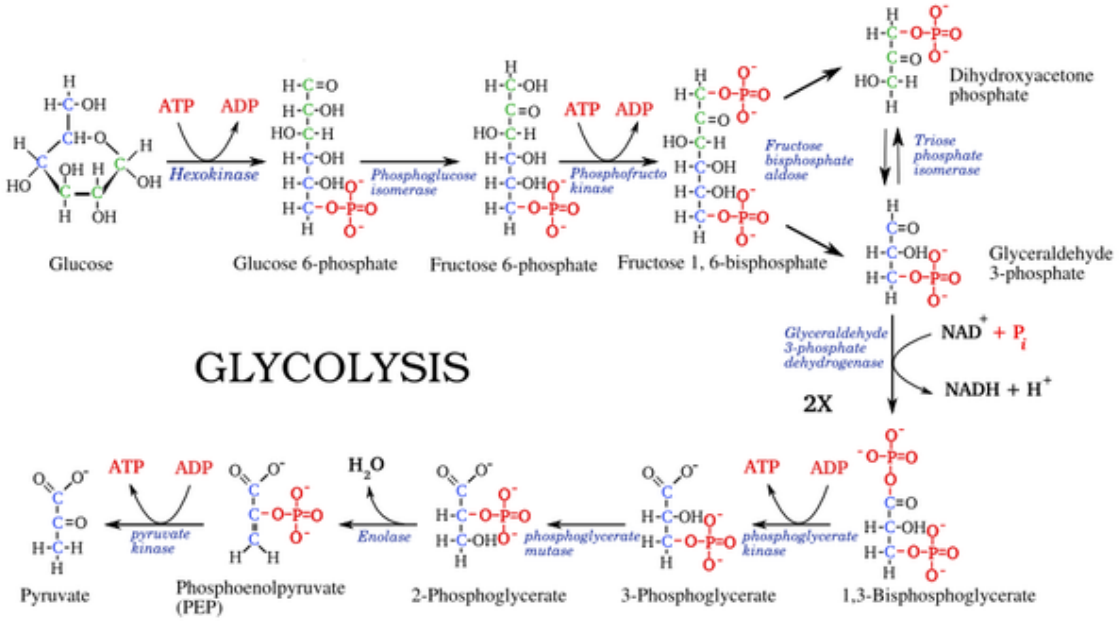


Illustration 26 It is a graphic scheme of the 10 reactions that take place in glycolysis. The first 5 reactions are called the preparatory phase in which 2 ATPs are spent to produce 2 Glyceraldehyde 3 phosphate, then the pay off phase produces 4 ATPs to supply again the first phase, a NADH and 2 pyruvate molecules. These reactions are produced in the cytoplasm. The blue and green C chains show where the C of the carbohydrates goes. The red molecules are phosphate groups from ATP. The blue names are the enzymes' names and the black ones are from the molecules.

After this pathway, 2 pyruvates are synthesized. These can go to different pathways:

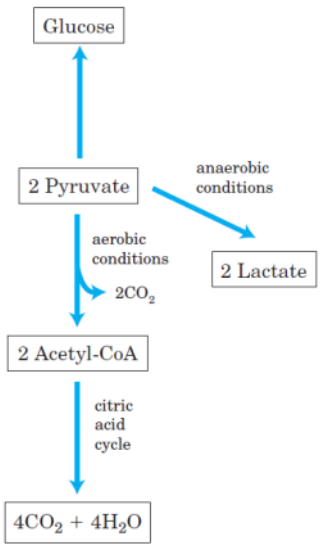


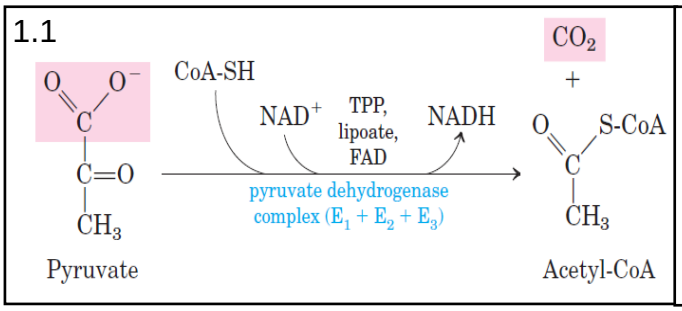
Illustration 27 There are 3 main paths where these pyruvates can end up:
-In need of glucose, they can go back to glucose through the 11 reactions of the gluconeogesis pathway which share most enzymes with glycolysis.
-In anaerobic conditions (absence of Oxygen), they can do the lactic fermentation and produce lactate using the NADH produced in glycolisis.
-In aerobic condition (presence of Oxygen), they can become acetyl-CoA and enter to the Krebbs Cycle or Citric acid cycle through the citrate enzyme.

3.4.2. Aerobic catabolism

Humans cannot live without oxygen, as aerobic metabolism (cellular respiration) is the only way we have to produce huge amounts of energy to sustain ourselves. This catabolic path is made of three pathways:

1. Oxidation of pyruvate into Acetyl-CoA and CO_2.
2. Citric acid Cycle or Krebs cycle.
3. Oxidative phosphorylation.

1.1



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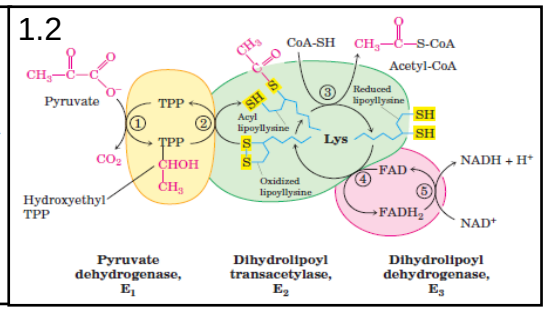

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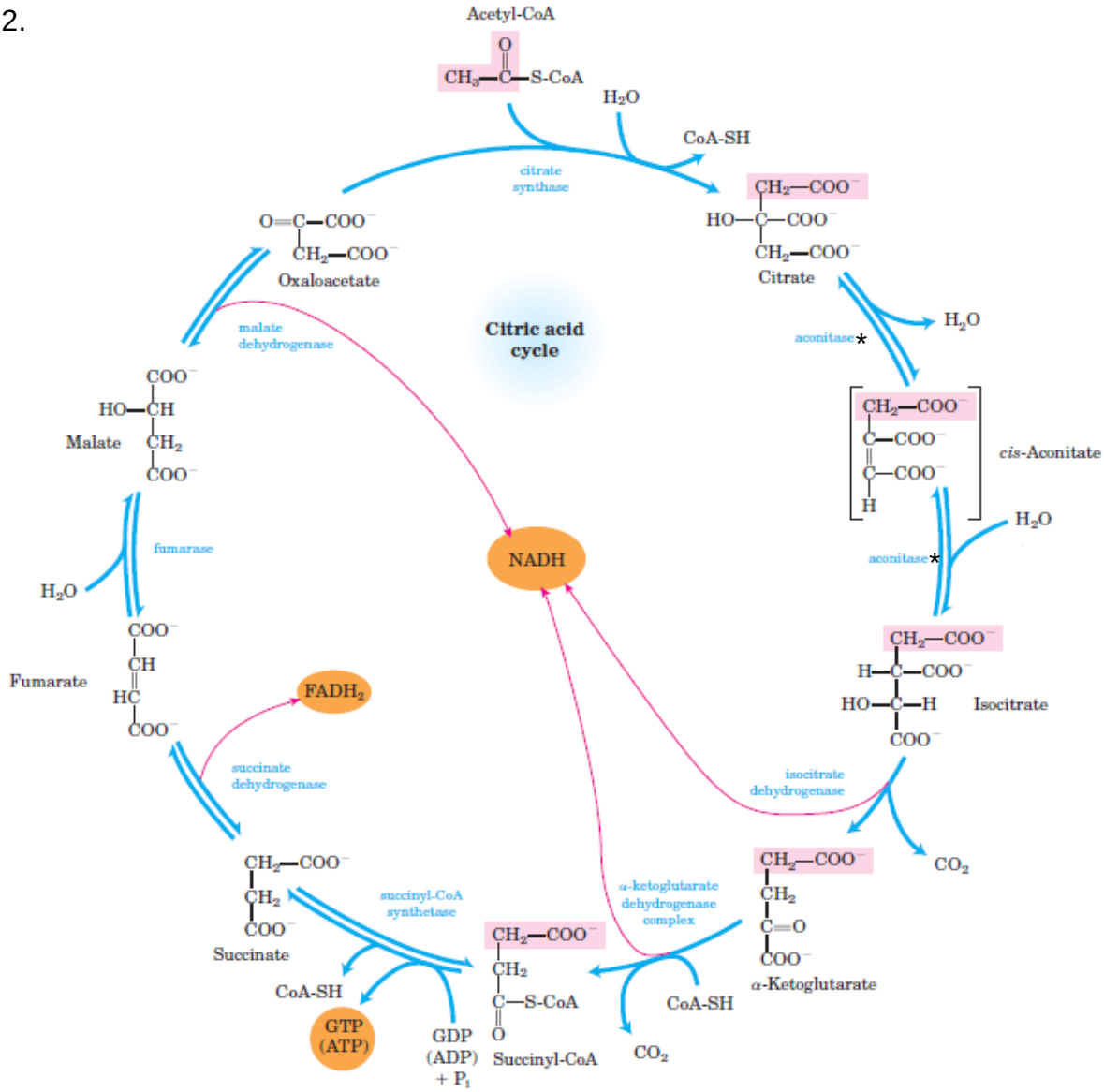


Illustration 28 These pictures show two pathways that happen in the mitochondria. The upper ones, how the pyruvate is oxidized into acetyl-CoA and the lower one how Acetyl-CoA enters the Citric acid cycle (CAC) to produce GTP, NADH and $FADH_2$.
1.1) The Pyruvate dehydrogenase complex uses 5 coenzymes (CoA-SH, NAD^+, FAD, lipoate and TPP) and pyruvate to produce Acetyl-CoA, NADH and CO_2. **1.2**) The complex in charge of the reaction is formed by three different enzymes Pyrucate Dehydrogenase (E_1), Dihydrolipoyl transacetylace (E_2) and Dihydrolipoyl dehydrogenase (E_3).
2.) CAC is a metabolic pathway made of 8 reactions (***** these are a two step reaction showed as two) which, catabolically speaking, their goal is to produce GTP, NADH and $FADH_2$ while oxidizing acetyl-CoA.

After the two first reactions the NADH and $FADH_2$ produced go to the oxidative phosphorylation pathway to produce 38 ATPs (3 ATPs for 1 NADH, 2 ATPs for 1 $FADH_2$ and an ATP for a GTP).

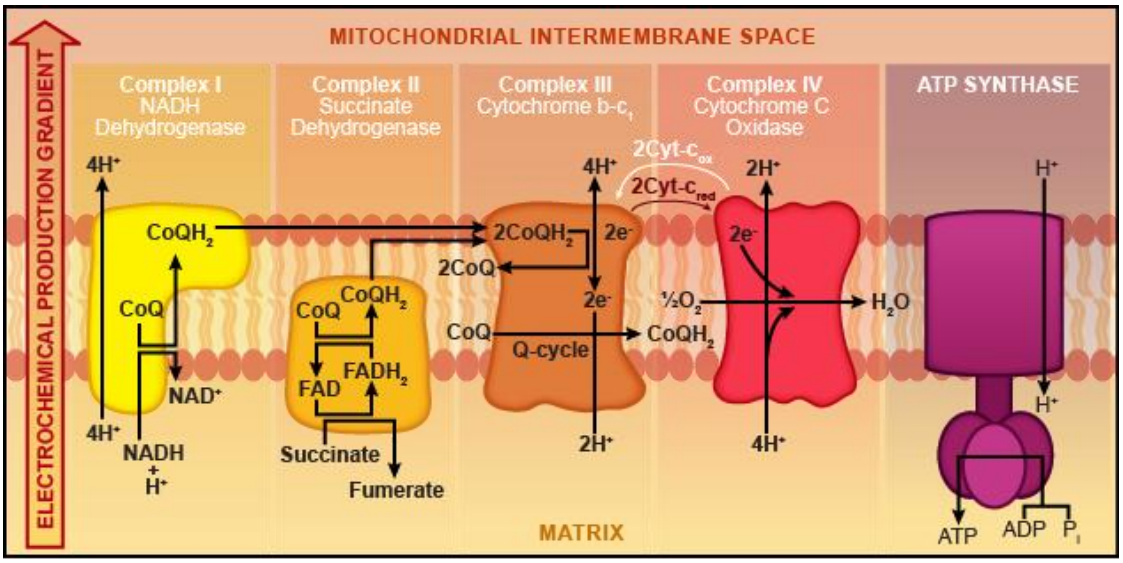


Illustration 29 is a graphical representation of the enzymes chain located at the internal mitochondrial membrane in charge of doing the last step of the respiration (aerobic catabolism). Each of the four complexes work together to deprotonate NADH and $FADH_2$ and pumping the H^+ to the intermembrane space to produce a chemiosmosis with the $[H^+]$ so they are pressured to enter the matrix through the ATP synthase rotating some enzymes from ATP synthase and producing ATPs.

3.4.3. Lactic acid fermentation

In anaerobic conditions many organisms and in humans some tissues (erythrocytes and skeletal mussels) do fermentations to produce energy. So after the glycolisis had produced 2 ATPs these cells turn the pyruvate into lactate.

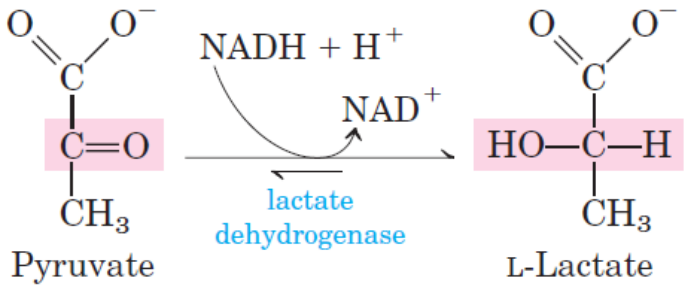


Illustration 30 is the graphical representation of the reaction that turns pyruvate into lactate using the NADH produced during the Glycolysis. In humans the lactate is send to the liver where it is recycled as new glucose.

3.4.4. Glutaminolysis

Cells need a lot of N to sustain amino acid and nitrogen bases anabolism. A N feeding pathways taken in account during the experimentation was Glutaminolysis the production of glutamate from Glutamine. After this, Glutamate can participate in many different reactions to become other amino acids or entering the Krebs cycle.

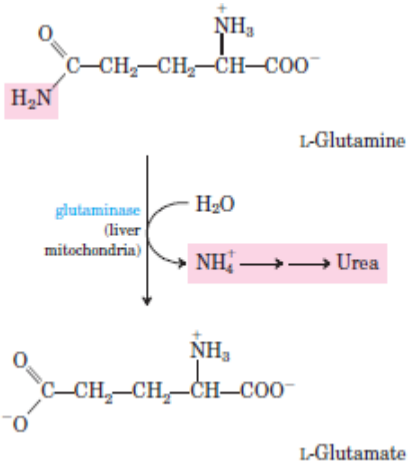
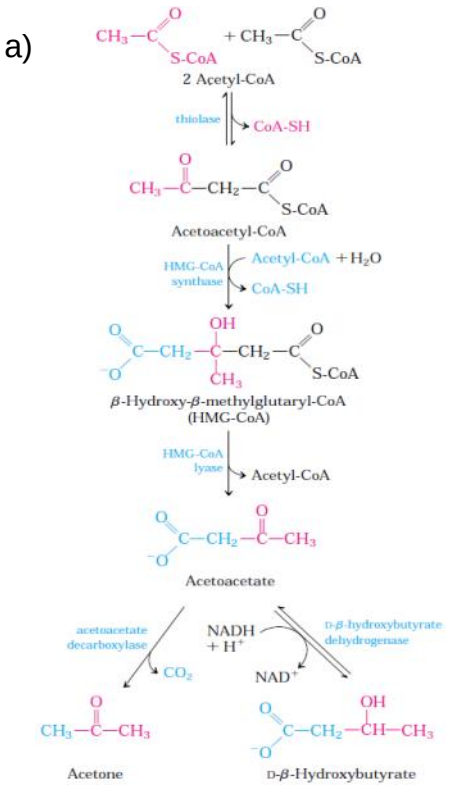


Illustration 32 shows the reaction performed by the glutaminase to produce glutamate. The ammonia ion (NH_4^+) produced must go to the Urea cycle (another pathway) because it is a neurotoxin that could damage ourselves but that we excrete through urine without taking any risk.

3.4.5. Ketone bodies

In most mammals, acetyl-CoA formed in the liver during oxidation of fatty acids can either enter the citric acid cycle or undergo conversion to the "ketone bodies," acetone, acetoacetate, and D-β-hydroxybutyrate (bodies is usually used with insoluble molecules although these compounds are really soluble in blood and urine). Once they are produced they are exported to other tissues were they will be converted into acetyl-CoA to oxidize them.



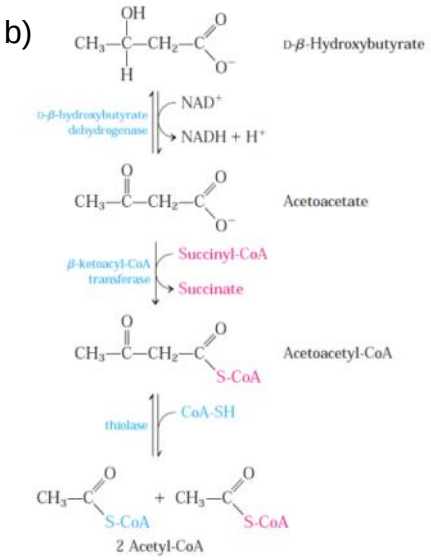


Illustration 31 These two reactions are the ones that interact with the ketone body used in the study (β-hydroxybutyrate). **a)** This one happens in the liver and produces the ketone bodies, then the compounds are transported to other tissues and **b)** become Acetyl-CoA again so it can become energy.

4. CANCER

4.1. INTRODUCTION

It is an illness suffered by 14.1 million cancer cases and 8.2 deaths in 2012 worldwide. In developed regions female breast, prostate, lung and colorectal cancer comprise half of the total incidence. [1]

4.2. HALLMARKS OF CANCER

Cancer is an umbrella term, a hypernonym, which includes a lot of different diseases from many points of view like the organs harmed, the genetic modifications that produce it, etc. However, there are some necessary functional capabilities implied to be called cancer that we use as hallmarks to recognise and catalogue this illness.

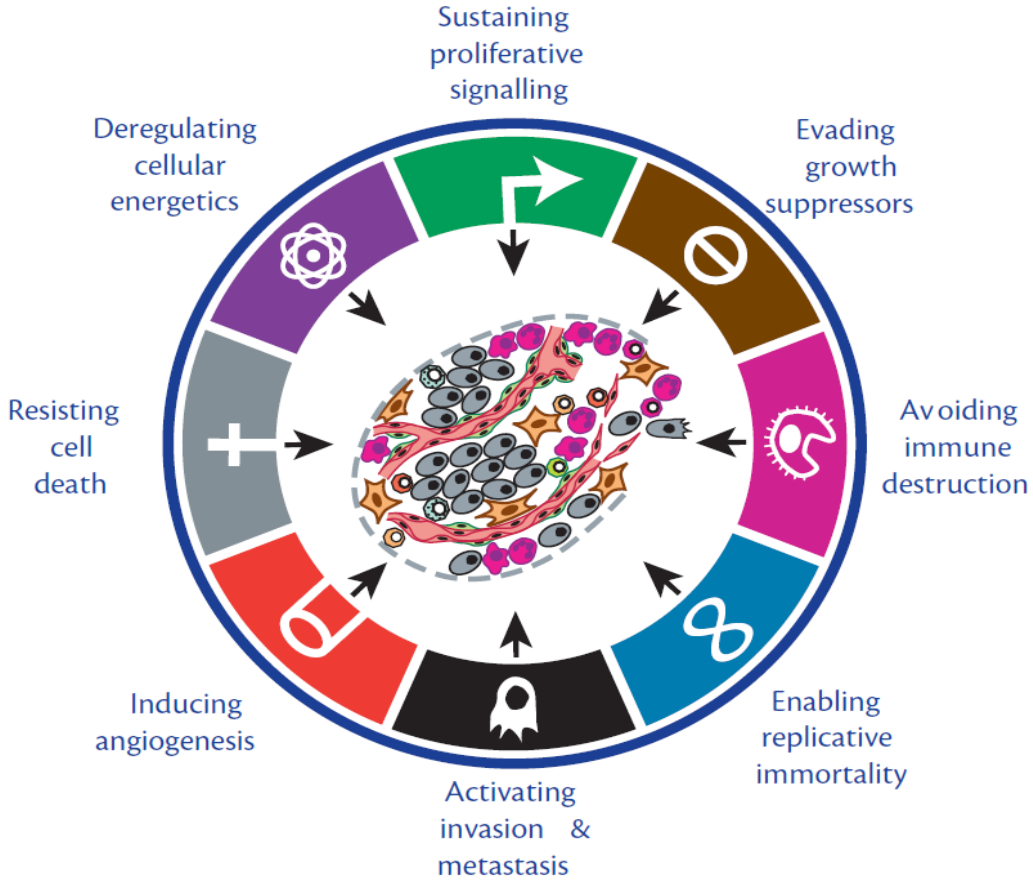


Illustration 33 The Hallmarks of cancer. Eight distinctive functional capabilities thought to be necessarily acquired during the pathogenesis pathways leading to most forms of human cancer. Some cancers may be more or less dependent on one of these hallmarks.

4.2.1. Sustaining proliferative signalling

The essence of a cancer is a deregulated cellular programme that instructs the cells to grow and divide at inappropriate times and places. This cellular programme is the cell signalling and is usually deregulated by oncogens.

4.2.1.1. *Cell signalling*

Cellular growth, metabolism, migration, differentiation and cell death are some of the multiple things regulated by the cell signalling. But how does it work?

Cell signalling is a complex network of electrical and chemical signals. In humans electric signals are only used for specific tissues (myocardium and brain), whose cells are connected with low-resistance channels like gap junctions.

This communication is simplified in three steps:

- Reception: A stimuli binds or reacts with a target receptor which is changed.
- Transduction: the change in the receptor starts a pathway in which different proteins send a message, normally to the nucleus. The cascade of interactions is accomplished usually by protein-protein associations and protein phosphorilations.
- Response: at the end of the path many things have been stimulated or inhibited showing a global response like apoptosis, cell growth, etc.

The molecules involved in this process can be nucleotides (cyclic ADP), lipids (DAG), amino acids (some neurotransmitters), proteins (kinases, transcription factors, ion channels, hormones) and inorganic ions (mainly Ca^{2+}).

Like in metabolism, to describe certain signalling group of interactions we use pathways.

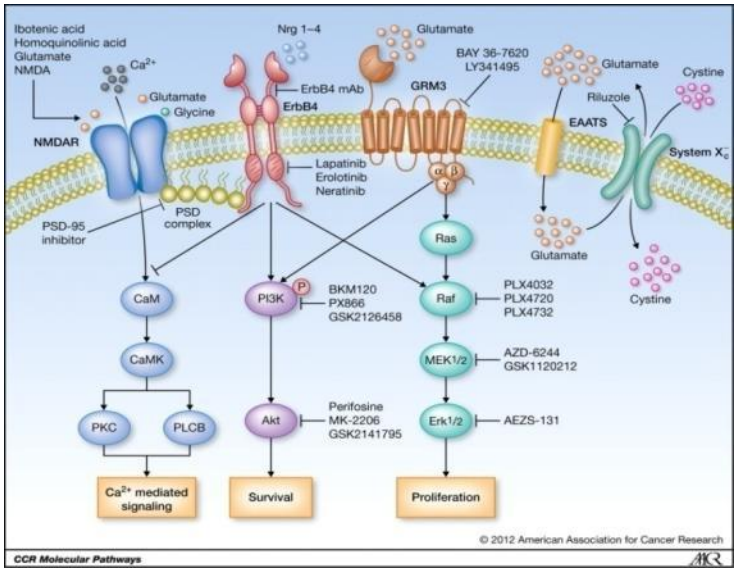


Illustration 34 This picture shows 4 different signalling pathways stimulated by glutamate in melanoma. The arrows show in which order they communicate and the lines with bar edges are inhibitors that could be used as treatments for this cancer inhibiting pathways like RAS-RAF-MEK-ERK which promotes proliferation.

4.2.2. Evading growth suppressors

Complementing the proliferative nature of the tumour, cancer cells can evade the effects of growth suppressors through different mutations and modifications of the braking systems of the cell division cycle. These brakes are embodied in retinoblastoma proteins (pRB) and “cyclin-dependent” kinase inhibitors.

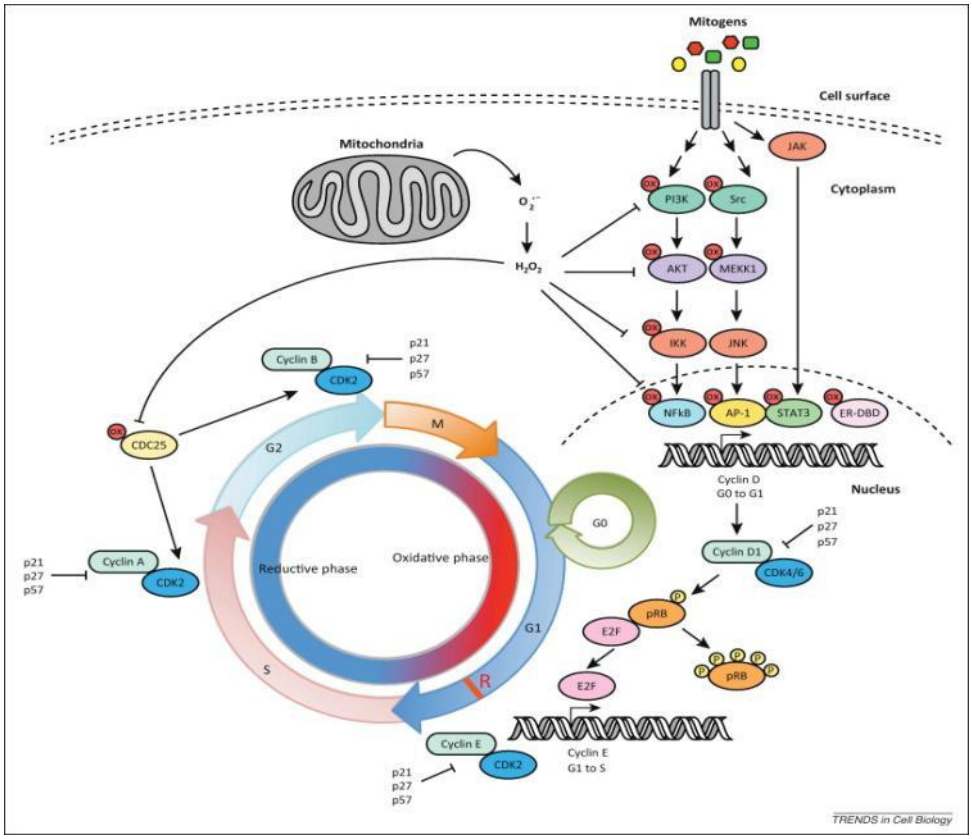


Illustration 35 shows the cell cycle redox regulation.

4.2.3. Resisting cell death

There are three programmes by which cells highly stressed may die:

- Apoptosis: programmed suicide of aberrant cells, it may happen there are severe genomic or physiological abnormalities.
- Necrosis: death caused by external factors. Death by necrosis results in a plasma membrane rupture that ends up in an inflammation that activates an immune response to repair the harmed zone.
- Hyper-activated autophagy: in severe conditions a recycling mechanism like autophagy can turn into a “cannibalistic” tool that turns the cell unviable.

For example the p53 is modified in approximately half of human cancers. That is because p53 pathway can induce apoptosis and, as it would constrain cell proliferation under normal conditions, in order to proliferate and grow they need to hinder or suppress that pathway.

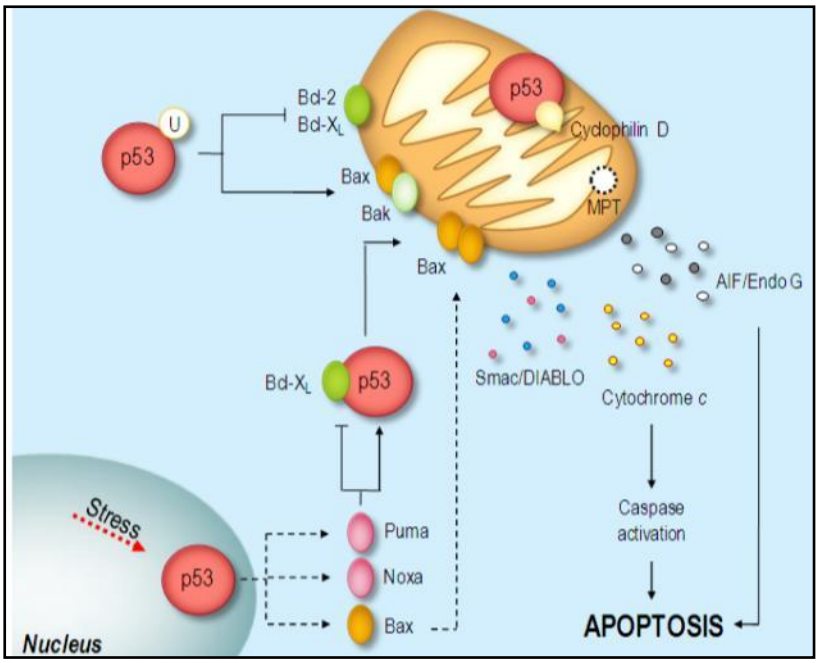


Illustration 36 This picture shows the role of p53 in terms of inducing apoptosis. The p53 from the nucleus will activate cytosolic p53 that will produce changes in Bax that will stimulate mitochondrial p53 to send chemical signals that induce its death.

4.2.4. Enabling replicative immortality

In a normal mammalian tissue, after each cell division (except in embryonic development) the telomeres, the nucleotides located at the edges of the chromosomes, are shortened. When the telomeres are highly cut off, the p53 mechanism is triggered. As we have seen p53 is usually modified in cancer, but as the damage produced in each cell division doesn't disappear it would reach a point where chromosomes were unsafe and unstable so the cell would die anyway.

Therefore, cancer cells should not develop and die at certain point, but they activate a telomere maintaining mechanisms that is used in embryonic development to avoid the barrier that supposes overly eroded telomeres. That means that cancer cells can replicate forever without suffering the "aging" of its cells. That is why there are thousands of samples in laboratories around the world of the same tumour like in the HeLa cell line case.

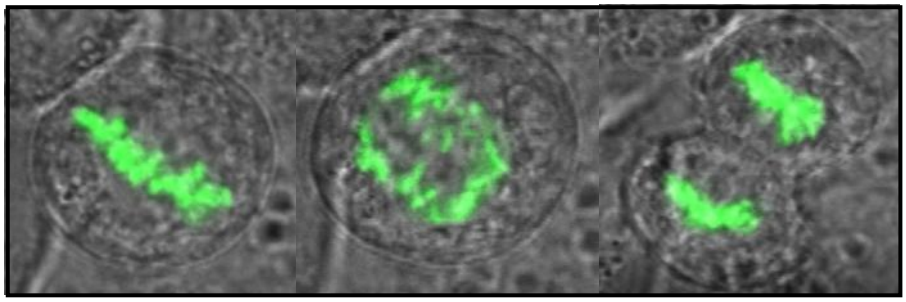


Illustration 37 Cervix cancer cells (HeLa) dividing. These pictures were taken at the CCiT.

4.2.5. Inducing angiogenesis

Our tissues require a steady supply of metabolites and a way to rid off wastes, this is pursued through vasculature (blood vessels). The main structures are produced during embryonic development through a process called vasculogenesis, but the expansion and remodelation of the vessels network is thanks to angiogenesis.

Therefore, angiogenesis is vital for neoplastic growth like in a tumour to survive, because many cells would die in absence of nutrients and in excess of metabolic wastes which would hinder the proliferation and the cellular development until they died.

Angiogenesis consists in series of steps leading from vessel sprouting, tube and lumen formation, vessel fusion and finally quiescence. It is being molecularly defined, but nowadays we have some anti-angiogenic drugs that have given good results.

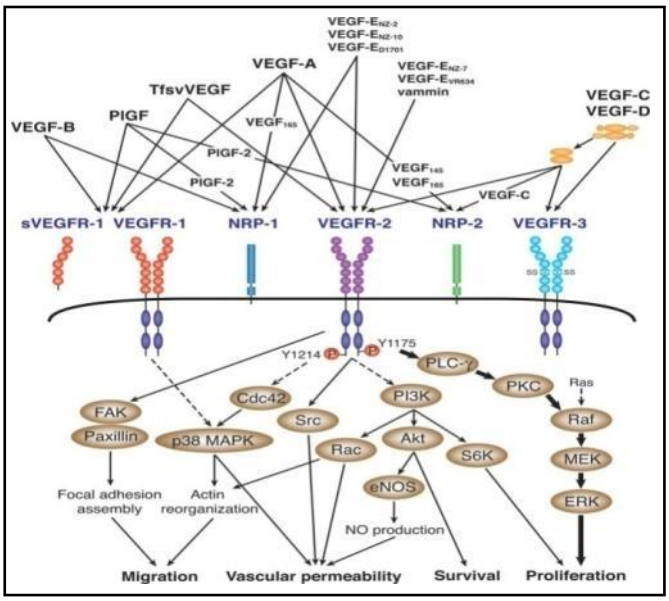


Illustration 38 VEGF/VEGFR signalling pathways of the growth factor VEGF targeted in all anti-angiogenic drugs.

4.2.6. Activating invasion and metastasis

We consider cancer when a tumour is malignant which means it has three conditions:

- Anaplasia: condition of cells with poor cellular differentiation, losing the morphological characteristics of mature cells and their orientation with respect to each other and to endothelial cells.
- Invasiveness: capacity of cancer cells to access to means of dissemination such as the lymphatic system and circulatory system (intravasation).

- Capacity to metastasize: when a tumoural cell is able to travel to another tissue from the primary tumour to another part (organ) of the body and then reproduces itself and grows as another tumour distant site from the one it came in first place.

If someone develops a tumour but it is not able to metastasize we say it is a benign tumour and it is not considered cancer.

4.2.6.1. Invasion

In order to access to a mean of dissemination, cancers grow killing nearby cells. For example, one known mechanism is through excreting protons (H^+) produced in glycolysis to activate apoptosis in other tissues through p53 which in cancer is mutated or even null.

Most of invasion and migration mechanisms are unknown despite the years of research we have spent trying to solve this mystery. But through *in vitro* assays, like the wound healing (Matrigel), gave us some clues about how it may work.

4.2.6.2. EMT and MET

Epithelial mesenchymal transition (EMT) and mesenchymal epithelial transition (MET) are two processes of phenotype variation in which cells change their gene expression in order to adapt to new conditions. When cells enter in a lymphatic or circulatory vein they need different abilities than when they have to adapt in a new tissue.

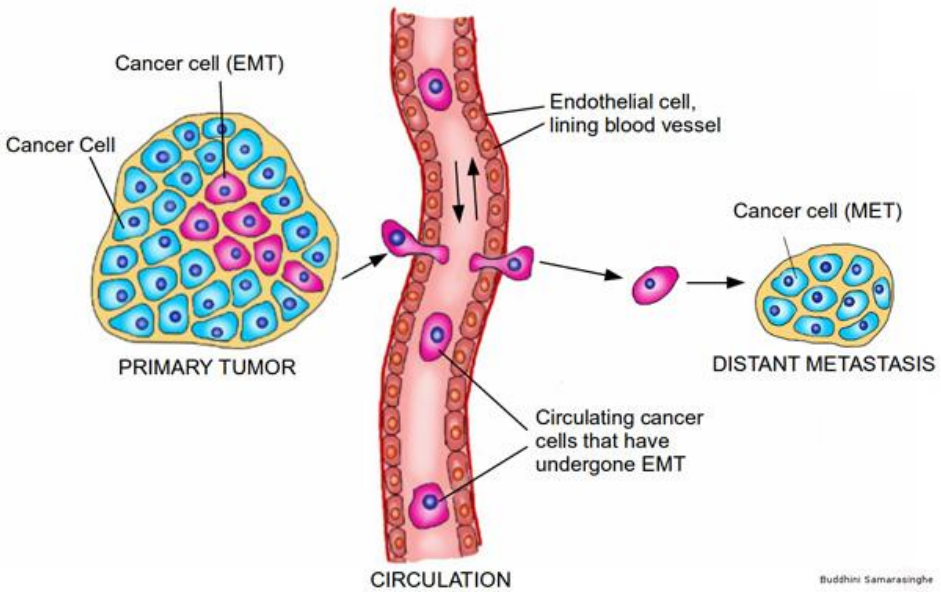


Illustration 39 This picture shows how the EMT happens in the tumour and then invades a blood vessel. This mesenchymal cell can travel and metastasize doing a MET in other tissues.

4.2.7. Deregulating cellular energetic and metabolism

Almost 90 years ago, Otto Warburg found that cancer cultures had a higher glucose uptake than its normal counterparts. This uptake and the following glycolysis proceed about ten times faster. Even in presence of O_2 which would sound counterintuitive as Oxidative phosphorylation is much more efficient. But we now know that this tendency to the lactic acid fermentation is because it helps to produce the cell building blocks much faster. That is why proliferative cells also pursue this “Aerobic glycolysis” in which CAC and Oxidative phosphorylation are not completely switched off just reduced.

Moreover, there are studies (for example putting cancerous tissue in glucose deprivation) that show that glutamine may be a good supplement for glucose as a fuel, as it is a precursor of lipids and amino acids; and it may also be a compensation mechanism if there was not enough glucose.

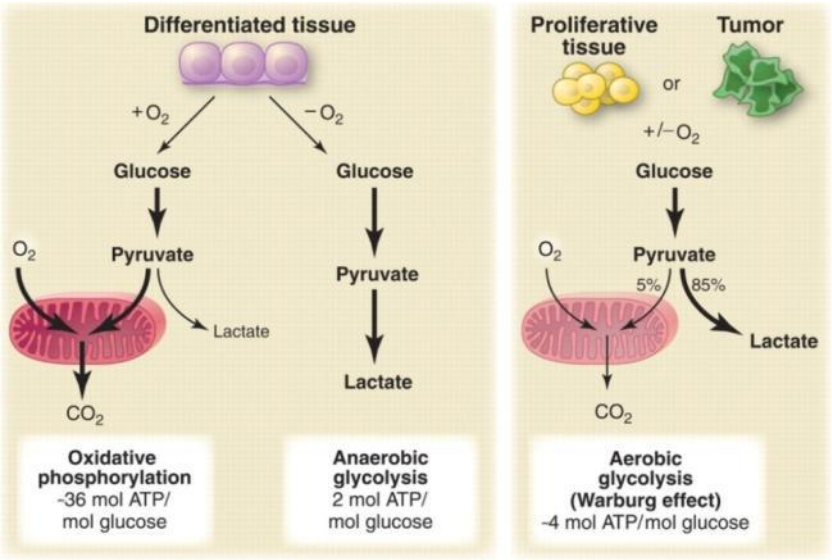


Illustration 40 This two pictures show the main catabolic pathways of differentiated tissues and proliferative or tumoural tissues

4.2.8. Avoiding immune destruction

The immune system is another barrier when trying to proliferate without control. Meanwhile the cancer doesn’t produce any strange membrane receptor the immune system will not activate any aggressive response. But in cancer produced by oncogenic viruses (20%) or when some tumours express membrane proteins from embryonic development (in which the immune system self-tolerance was not active) the T cells may try to rid off them.

In colorectal carcinoma, it was seen that similar grade and size tumours with different concentrations of Cytotoxin T-Lymphocytes reacted different to treatment, having a better prognosis those with higher concentrations.

4.3. COLORECTAL CANCER

Colorectal cancer (CRC) is the third most common in men and the second for women in the world [1], this means 1.36 million new cases per year and 694,000 deaths attributed to this disease. Moreover, it is the cancer where the cell lines I used in the experimentation part come from.

It is a cancer produced on the walls of the large intestine (colon) or on the walls of the rectum. This kind of cancer usually metastasis first to lymph nodes and then if it is not treat it usually metastasize further to the liver and the lungs.

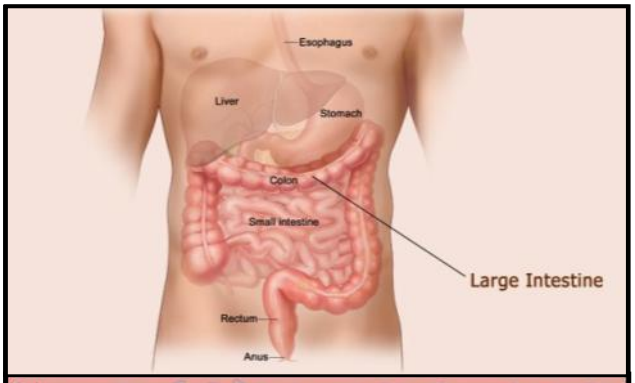


Illustration 41 shows some organs of the digestive system and their location inside our body. It is highlighted the Colon or Large intestine which is marked with a line.

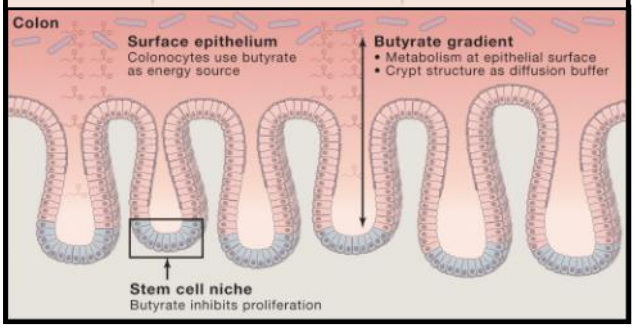


Illustration 42 shows the structure of the colon walls which is an epithelium tissue folded to form crests which are called crypts. These crypts are made of stem cells at the bottom and as they go up they differentiate more.

Approximately 75% of all the CRC cases are sporadic, so they are not related to any hereditary element. In addition, there are many studies that have found many risk factors in our lifestyle culpable of the 50% of the sporadic cases, so we should avoid them or visit once a year the doctor for a checkout to find it and treat it in its firsts stages. Some of these risk factors are:

- High ingest of red meat due to the animal fat that increases the bile acid that reaches the colon and that would act as a mutagen. Moreover,

nitrates and nitriles used for processed meat conservation are also bad.

- Cigarette smoking. Those who smoke not only have more chances to suffer a CRC; they also have a higher mortality.
- Alcohol. There are ongoing studies with low intake, but it is already demonstrated that consuming more than 4 standard drinks per day will increase your chances to develop it. [15]

4.4. CANCER THERAPIES

Cancer is so complex because it has to stand and get over many human defence systems, like the immune system, as we have seen in the hallmarks. That causes a huge variety of problems when trying to treat it. But nowadays we have two really effective methods: Chemotherapy and Radiotherapy. Moreover, there is a treatment in process that has really good perspectives, Immunotherapy.

But as having a cancer means much more than just one or some tumours, they weaken you or even harm you in many ways like cachexia. So when someone is suffering cancer, we try to treat them from a variety of point of view. Those are just complementary, in order to cure, surgery, chemotherapy or radiotherapy is unavoidable. But there are studies, as in many different illnesses, trying to support these treatments with diets, activities... to create favourable conditions to get over it or difficult conditions for the cancer to develop. The main treatments are:

- Chemotherapy: Consists in administrating intravenously a combination of drugs that weaken or damage cells in general, but, especially, those that are cancerous.
- Radiotherapy: Consists in irradiating a punctual tumour with ionizing radiation to strongly damage the DNA so the cancerous cells die.
- Immunotherapy: there are two different treatments that are being studied. One focuses on activating our immune system to kill the cancerous cells, the other, on creating antibodies bonded to drugs that would attach to the cell and release the drug inside.[2] [10]

5. BIOINFORMATICS

5.1. INTRODUCTION

Bioinformatics is a tool that appeared in the XX's century with the creation of computers. It is not a science; it is the programming application of different sciences like biochemistry.

We consider Margaret Oakley Dayhoff[3] as the mother of bioinformatics, as she was the first who created a sequence database using the basic technology of her time. Once you are working with sequences from all the nucleic acids, or amino acids; in a genome or protein, respectively; you are working with a whole system whose field is named with the suffix "-omics". For example genomics, proteomics, metabolomics.

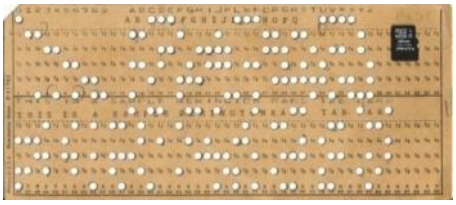


Illustration 43 is a punched card. It is a rudimentary "memory card" used with the firsts computers; one like this may have been used by Margaret Oakley. In the picture, we can see a 16GB microSD which can store as much data as 236,993,710 Remington Rand cards like the one on the picture.

Technology has arrived to a point where sequencing genomes, proteins, and gathering many types of data is really easy, so now, bioinformatics has taken a main role in biology as it helps to arrange and manage the data through software and algorithms. An example of that is the NCBI, a scientific database of databases in which you can find many different things, from DNA, RNA and protein sequences to scientific studies.

5.2. METABOLOMICS

Metabolomics is the field inside metabolism that studies all the chemical reactions from a systemic point of view, in which we study every reaction at the same time. Unlike formerly biology in which we studied separated reactions or enzymes.

[3] Margaret Oakley Dayhoff (1925 –1983) was an American physical chemist professor at Georgetown University.

5.2.1. Systems Biology

Systems Biology is the scientific discipline that studies the systemic properties and dynamic interactions in the biological object, be it a cell, an organism, a virus, or an infected host, in a qualitative and quantitative manner and by combining experimental studies with mathematical modelling. Scientist can describe inner processes of star thousand light years away with great accuracy. But how a tiny cell under our microscope grows and divides remains puzzling in many ways. We see kids growing, people aging, plants blooming. But do we understand how processes of life work? [16]

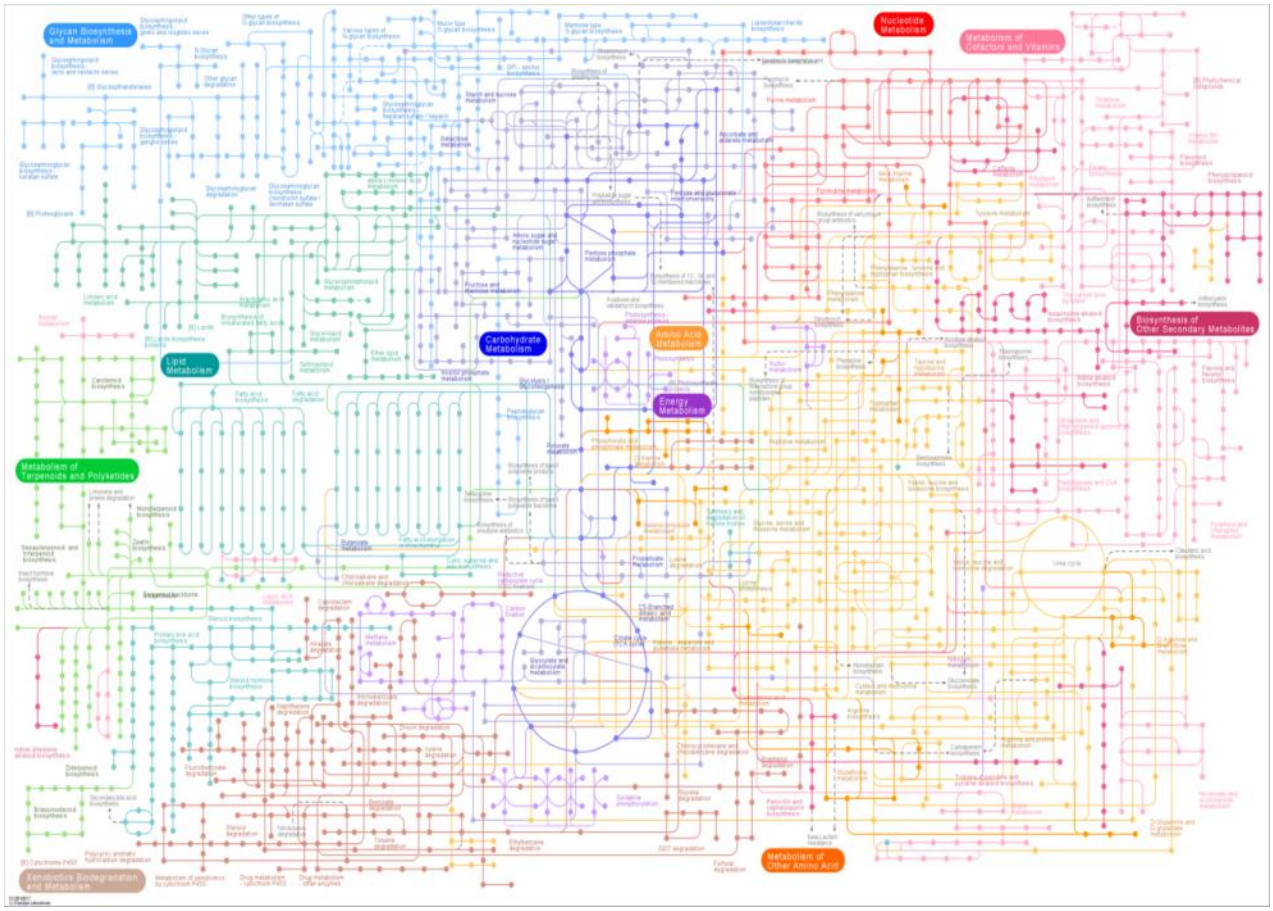


Illustration 44 This is a metabolic map that contains most human metabolic pathways (1 colour per pathway) that shows how complex and large metabolism can be and how unmanageable without computational technologies it is to approach as a whole.

In a broad sense, a model is an abstract representation of objects or processes that explains features of these objects or processes. A well-known and practical way to express a model is through mathematics, moreover, nowadays that we have incredible calculation machines, computers.

$$\Delta\vec{r}=\vec{r}_f-\vec{r}_i \qquad \vec{v}=\frac{d\vec{r}}{dt} \qquad \vec{a}=\frac{d\vec{v}}{dt}$$

Equations 2 Those equations are a model of classical kinematics, a mathematical way to explain the movement of particles. Through the mathematical equations that compose a model we can describe its displacement, position, speed, acceleration and predict them.

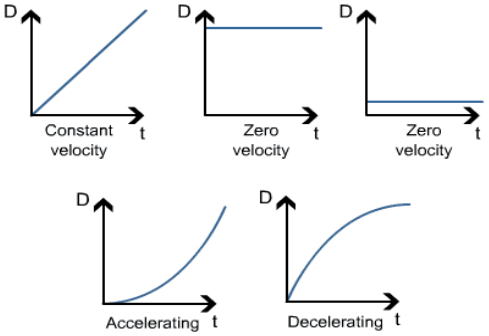


Illustration 45 Those are different displacement (D) and time (t) graphs that change depending on the velocity in which a particle is moving. Expressing it graphically let us comprehend better what it is inherited in the calculations despite we lose some information in exchange.

As we use a model to express the movement of any object, we are going to do different calculations in order to understand and describe the metabolism of an organism. These equations will be different, but they will be used the same way, for example to do predictions or to optimize.

5.2.2.1. *Advantages*

Models are fundamentally based on real experimentation, so they are really dependant on the quality of those in which it is made by. Nevertheless, modelling compared with further experimentation is better than only purely experimental studies:

- Modeling drives conceptual clarification. It requires verbal hypotheses to be made specific and conceptually rigorous.
- Modeling highlights gaps in knowledge or understanding. During the process of model formulation, unspecified components or interactions have to be determined.
- Modeling provides independence of the modeled object.
- Time and space may be stretched or compressed *ad libitum*.
- Solution algorithms and computer programs can be used independently of the concrete system.
- Modeling is cheap compared to experiments.

- Models exert by themselves no harm on animals or plants and help to reduce ethical problems in experiments. They do not pollute the environment.

- Modeling can assist experimentation. With an adequate model, one may test different scenarios that are not accessible by experiment. For example, one may cause precise perturbations without directly changing other system components, which is usually impossible in real systems. Model simulations can be repeated often and for many different conditions.

- Model results can often be presented in precise mathematical terms that allow for generalization. Graphical representation and visualization make it easier to understand the system.

- Finally, modeling allows for making well-founded and testable predictions. [16]

5.2.2.2. *Types*

Metabolic modelling can mainly be pursued in two different ways:

-Constraint-based modelling: This kind of mathematical modelling assumes steady state.

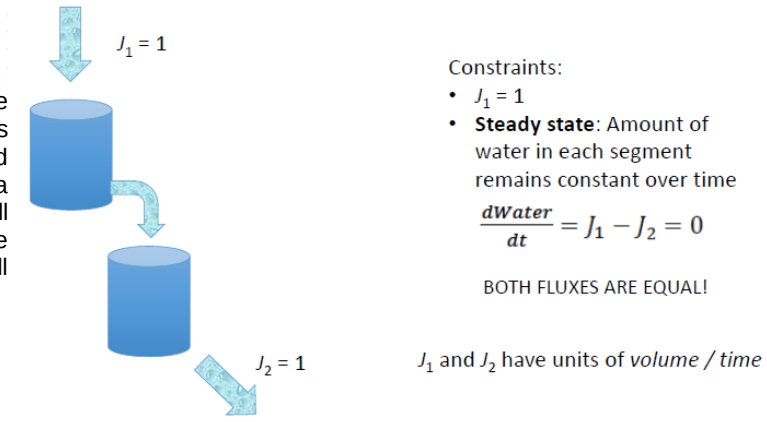


Illustration 46 Is an explanation of the Steady State using water tanks. In this example the flux of water would represent the flux of metabolites in a pathway and the constraint (J_1=1) will establish the flux of water as 1 so the amount of water (in this example) will always be one.

Steady state is a term that can be explained really easily with the picture above. If you have a cup with a hole, the cup will end up empty. However, if you fill it at the same speed it will not end up empty although it is losing the same amount of liquid than in the first case.

-Kinetic modelling: Doesn't assume it, so it is base in different equations and even data as it needs to supply all the information and calculations the

constraints where ignoring. This kind of modelling is even more complex so we are not to describe it in more detail.

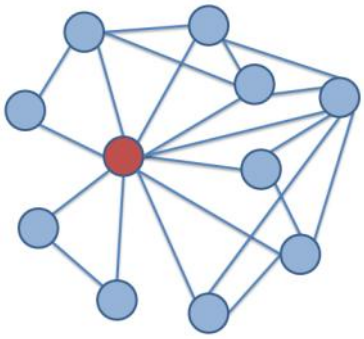


Illustration 47 This is a network, the circles are the nodes and the lines are the edges.

5.2.2.3. *Networks*

A network is a graph formed by edges and nodes. It is used in Systems Biology as a conceptual and graphical way to express different interactions. In metabolomics, the edges represent the enzymatic reactions and the nodes the metabolites.

Different metabolic examples of this kind of graph are throughout the second chapter (metabolism pathways) were each pathway is represented as a graph. This can be represented as Stoichiometric matrixes.

6. USING SCIENTIFIC METHOD

6.1. INTRODUCTION

The scientific method is a list of instructions developed a couple centuries ago, in which modern science is strongly founded nowadays. These instructions have been tested and changed by many scientists and philosophers in order to achieve a rational empirical method for any research done.

Diagram 4

Throughout this chapter you will see the different steps of my research project following the same structure.

6.2. ASK A QUESTION

"Does a ketone body like β-hydroxybutyrate affect the metabolism of a Colon Cancer and its metastasis?"

6.2.1. Why this specific question?

I wanted to study metabolism in a Systemic approach comparing responses to different environments inspired by a bioinformatic experiment I did on a science course.

I got the opportunity to work in a lab at the UB, so I wanted to take advantage and do something *in vitro*. However I only could work with the material of their lab, like the colon cancer model. But I didn't want to do an experiment they already had done, I wanted to do something new.

Firstly, I mentioned palmitate due to an awarded study I had seen [4], but I preferred to do something new, so the PhD student's advice was to try butyrate or β-hydroxybutyrate. Butyrate is a really common compound in the bowel, as our bowel's microbiome produces it a lot. Butyrate is also a huge differentiation factor, but as it is only present in the bowel, not in the blood I preferred to test β-hydroxybutyrate as it is present in our blood, especially in case of fasting of ketogenic diet.

6.3. BACKGROUND RESEARCH

Every time you want to do an experiment you must look for information related with your experiment. Maybe someone has already done it, or in similar conditions so you can get a lot of data before even starting. All the information about the Cancer Model is explained in the Cancer chapter at each cell line's part, the information about the β-hydroxybutyrate is the following:

6.3.1. B-hydroxybutyrate

The condition I applied to the experimentation was the presence of β-hydroxybutyrate. It is the major form of ketone bodies, so it is useful to look for its effects and properties as it is produced in the liver during fasting, ketogenic diet (low in carbohydrates) and in diabetes.[7] There have been many studies

that show that different tissues are able to metabolize them correctly, but it seem that in cancerous tissues although they are able to obtain energy [6] [9] [8], it is not metabolized as it should and it could even inhibit the growth of metastatic cells.[5]

6.3.2. Cell Lines

The model of colon cancer I used is based on three established cell lines: SW480, SW620 AND SW620-LiM2

- SW480: adenocarcinoma that has been found the following properties. It is the cell line with the highest volume and the one that proliferates the least. Moreover, it is the most glycolitic line and the one that consumes more glutamine in relation to glucose. Therefore, it is the most dependent to them. They survive the least under glucose (Glc) and glutamine (Gln) deprivation; half of them undergo apoptosis in both deprivations. But those who survive proliferate without cell cycle arrest. Although coming from the primary tumour, it shows some invasive properties.
- SW620: first lymphatic system metastasis. It is the cell line with the least volume, so the one that proliferates the most. In addition, it is a bit metastatic and as a metastatic cell its metabolism is more flexible and can survive at Glc and Gln deprivation arresting the cell cycle, a possible strategy to survive in the vasculature. At Glc and Gln titration Oxygen Consume Rate (OCR) increases. It also increases in presence of palmitate. They only undergo apoptosis under Gln deprivation, not under Glc.
- SW620-LiM2: highly metastatic line obtained from injecting a metastatic line from SW620 in mice [5]. It is the most metastatic cell line. As a metastatic cell its metabolism is more flexible and can survive at Glc and Gln deprivation arresting the cell cycle, a possible strategy to survive in the vasculature. At Glc and Gln titration Oxygen Consume Rate (OCR) increases. It also increases in presence of palmitate. They only undergo apoptosis under Gln deprivation, not under Glc (like in sw620).

6.4. HYPOTHESIS

I have developed a couple of hypothesis for the *in vitro* part and a third for the *in silico*:

1. – As it is a molecule that we use in fasting to get energy, the cancer cells will use it to grow.
2. – Despite the similarities between butyrate and β-hydroxybutyrate, I suppose that it won't differentiate the cells as they both have different metabolic pathways.
3. – The metabolic model using experimentation data will be enough accurate to show differences between the cell lines.

6.5. EXPERIMENTATION *IN VITRO*

Once I have learned about what I wanted to investigate, it was time to finally design the experiment, the variables, budget, and much other stuff, that as I had never studied experimental design it would have been impossible without the guidance of the PhD student that was responsible of me in the labs.

Now that we knew what to do and how, it was time to start. The procedure is explained with detail in the protocols and documents at the annexes.[4] Even so, I will overview the things done between 3rd and 21st of July.

6.5.1. Preparing 6-wells plates

It was the first day I went to the UB's lab, although the PhD student, had shown me the facilities before. We discussed the different things we had to prepare, such as the tools and reagents for the experiment.

[4] The first week protocols are in the annexe: III page XIV and other useful documents like the culture's room rules and COBAS MIRA PLUS protocols can be found in the annex IV from the page XVIII.

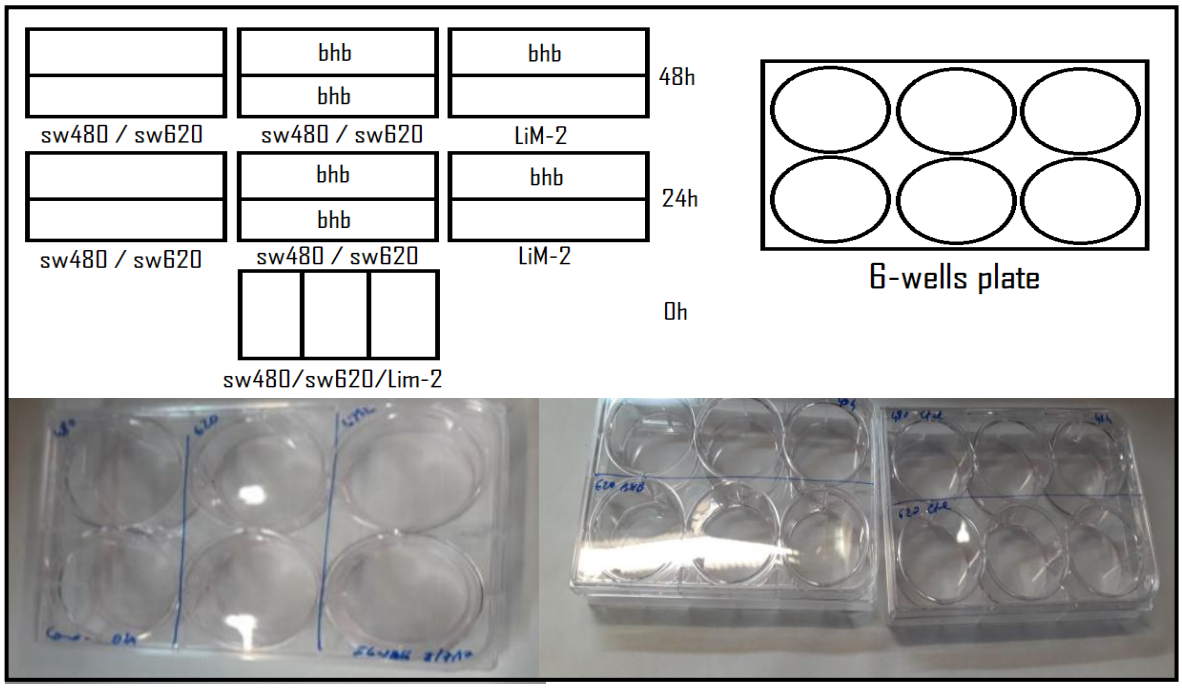


Illustration 48 Representation and pictures of the six-wells plates used and how it was organized. At the upper side we can see the wells and the organizations of the three cell lines, times (0, 24 and 48 hours), and condition applied (presence of bhb). Under the scheme we can see two pictures: the one in the left is the 0h plate and the two on the left are the 48h of the SW480 and SW620 cell lines with and without bhb.

Once we had decided all, we went to the culture's room to prepare the cell lines for the experimentation, which should be done in sterile conditions. (PROTOCOL 1) There we took the 3 cell lines checked if they had spread enough through the plate and we seeded into two different plates per cell line. One is a 100mm plate to store the cells, so they can be used in other experiments and the other is to let them adapt on the surface of 6 wells plate (Eukaryotic cells tend to stick on plates with proteins, when you change their plate you must break these attachments so they need time to produce them again).

$$100mm\ plate\ cell\ dilution\ to\ count\ cells\ =\ 1/20$$

$$sw480\ cells\ counted = 171{,}100\frac{cells}{\frac{1}{20}mL} \rightarrow 30mL \times \frac{150{,}000cells}{2mL} \times \frac{1mL}{3{,}422{,}000cells} = 657\mu L$$

$$sw620\ cells\ counted = 199{,}300\frac{cells}{\frac{1}{20}mL} \rightarrow 30mL \times \frac{200{,}000cells}{2mL} \times \frac{1mL}{3{,}986{,}000cells} = 754\mu L$$

$$LiM2\ cells\ counted = 277{,}400\frac{cells}{\frac{1}{20}mL} \rightarrow 30mL \times \frac{200{,}000cells}{2mL} \times \frac{1mL}{5{,}548{,}000cells} = 540\mu L$$

Equation 3 Calculations to control how many cells we seeded.

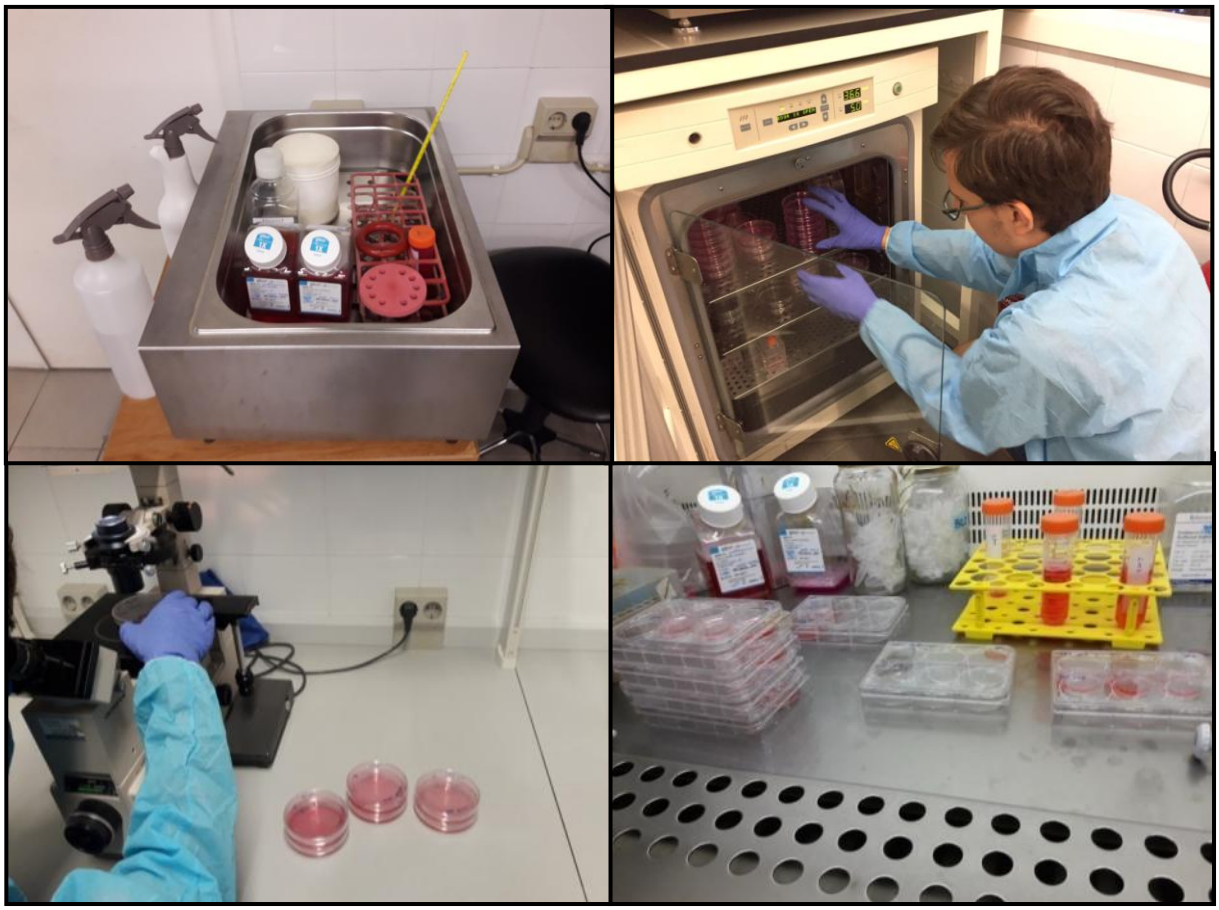


Illustration 49 Pictures working on culture's room. The upper left one shows the trypsin and the medium in the bain-marie. The upper right one shows how I am taking out the 100mm plates. The lower left one was taken while checking the proliferation of the lines. The lower right one was while preparing the 6-wells plates.

In the afternoon, after having lunch with all the lab partners, we planned the experimentation schedule and we seed some more cells for other experiments as a way to practice this procedure.

6.5.2. 0 hours cultures

The next day in the morning, I calculated and weighted the β-hydroxybutyrate grams I needed to prepare a medium with a concentration of 5mM. Some of this was stored for metabolites measurements and the other brought to the culture's room for the experimentation.

$$Maximum\ solubility\ of\ \beta-hydroxybutyrate\ (bhb) = \frac{1g\ bhb}{10ml\ H_2O} \rightarrow \frac{1g\ bhb}{0.01L\ H_2O} \times \frac{1mol\ bhb}{126.09g\ bhb} = 0{,}793M$$

$$1mL \times \frac{0.5\ mol\ bhb}{1000mL} \times \frac{126.09g\ bhb}{1mol\ bhb} \times \frac{100g\ commercial\ bhb}{98g\ bhb\ pure} \times \frac{1000mg}{1g} = 64.3mg\ commercial\ bhb$$

$$60mL\ medium \times \frac{5mol\ bhb}{1{,}000{,}000mL\ medium} \times \frac{1000mL\ stock}{0.5mol\ bhb} = 0.6\ mL\ \text{stock}$$

$$bhb\ weighted = 70mg$$

$$70mg\ commercial\ bhb \times \frac{1g\ com.bhb}{1000mg\ com.bhb} \times \frac{98g\ bhb}{100g\ com.bhb} \times \frac{1mol\ bhb}{126.09gbhb} \times \frac{1000mL\ H_2O}{0.5mol\ bhb} = 1.088\ mL\ H_2O$$

Equation 4 Calculations to prepare the 5mM β-hydroxybutyrate (bhb) medium.

Then I dissolved the compound on the medium so I could have 60 ml of each medium one with β-hydroxybutyrate and the other without it. Then we went to the culture's room to count the 0h cells and to add the medium to the 24 and 48h cell lines.

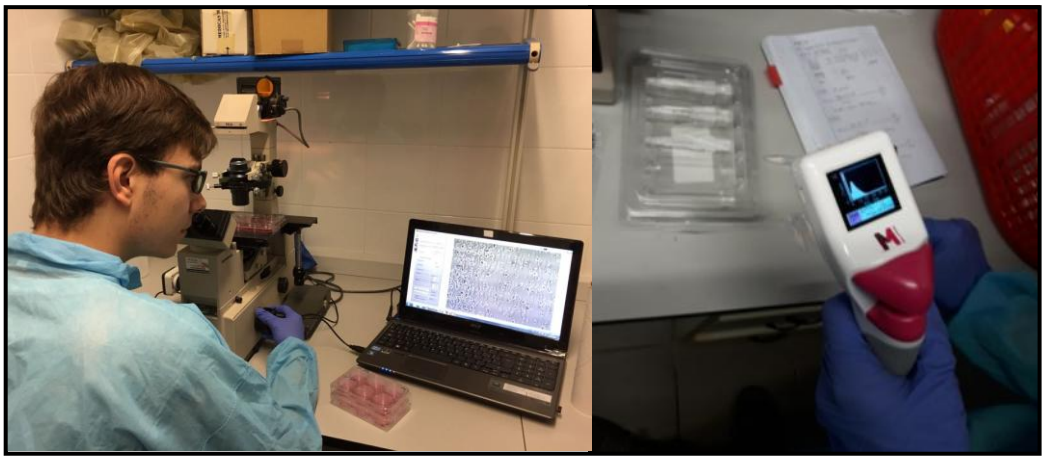


Illustration 50 Pictures working at culture's room. The left one was taken while taking pictures of the cultures. The right one shows the Sceptre cell counter's screen with the population and the volume of the cells of one sample.

6.5.3. 24 and 48 hours cultures

We did the same procedure both days (Protocol 2), it was basically taking pictures of the cells, counting the populations and storing the mediums of each so we would be able to measure it further. The cells were also stored because they can be used for further experimentation like SDS-PAGEs to look for

hallmark proteins.

During the afternoons of these last 3 days I started to look for information on the PubMed's platform and through the numerous biochemistry books around the lab and I also redacted the experimentation protocols of the processes completed until that moment.

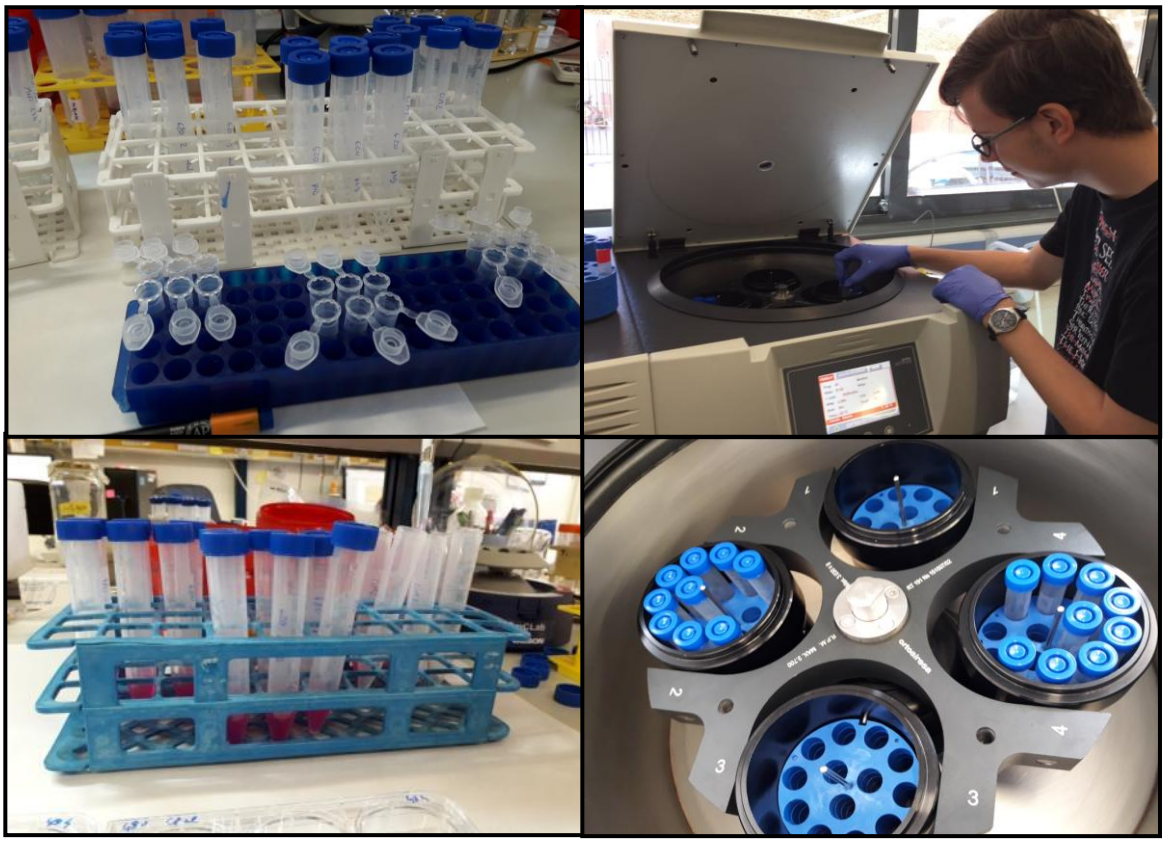


Illustration 51 Pictures from the 5th and 6th of July. The left pictures show the corning tubes and eppendorfs used to count and store the medium. The right pictures show the centrifuge and the distribution of the corning tubes inside it, which is very important to avoid any accident).

6.5.4. Spectrophotometry analysis

The next week I had a Bioinformatics course in the UAB, so we agreed to continue the one after that, and as all the samples were stored inside the fridge or the freezer there was no problem in waiting to analyze. During that week, the PhD student had a student preparing him for the final degree's project that also did some cell cultures so all the calculations for this week experiment had to be done for both samples as it was more efficient than preparing reagents for each

one's project.

$$Dead\ volume\ \boldsymbol{R} = 5ml\ \boldsymbol{SR1}\ and\ \boldsymbol{SR2} = 500\mu L$$

$$Lactate\ reagents: \qquad \boldsymbol{R}\ 250\frac{\mu L}{sample} \rightarrow 1mL\ Hydrazine\ 0.2M + 100\mu L\ NAD^+$$

$$\boldsymbol{SR1}\ 7\frac{\mu L}{sample} \rightarrow 100\mu L\ LDH + 200\mu L\ (NH_4)_2SO_4\ 3.2M$$

$$\boldsymbol{SR2}\ 10\frac{\mu L}{sample}\ H_2O\ milliQ$$

$$\sum Samples = 66 \qquad Lactate\ protocole\ requieres\ 3\ replicates$$

$$\boldsymbol{R} = (66 \times 3 \times 250)\mu L + 5000\mu L = 54mL\ \approx 60mL\ We\ will\ do\ 100mL$$

$$100mL\ R \times \frac{1mL\ Hydrazine}{1.1mL\ R} = 90.9mL\ Hydrazine \quad 100mL\ R \times \frac{0.1mL\ NAD^+}{1.1mL\ R} = 9.09mL\ NAD^+$$

$$\boldsymbol{SR1} = (66 \times 3 \times 7)\mu L + 500\mu L = 1{,}886mL\ \approx 2mL\ SR1$$

$$2000\mu L\ SR1 \times \frac{100\mu\ LDH}{300\mu L\ SR1} \approx 667\mu L\ LDH \quad 2000\mu L\ SR1 \times \frac{200\mu L\ (NH_4)_2SO_4}{300\mu L\ SR1} \approx 1333\mu L (NH_4)_2SO_4$$

Equation 5 Calculations used to prepare the reagents for the lactate procedure.

That week basically consisted in doing spectrophotometry analysis of the mediums to find the concentrations of Glucose (Glc), Lactate (Lac), Glutamine (Gln) and Glutamate (Glu).

The first day we just prepared the different reagents we would need to find each molecule concentration.

We started with the glucose analysis, which lasted all the morning. Glucose spectrophotometry is the easiest one and it usually works without any problems, this method is used for example in glycaemia analysis.

The next day we did it with lactate, which, as I was warned, was a tedious procedure, because the reaction used produced gas bubbles which usually hindered the light detection. That caused many second and even third analysis to ensure each result was correctly measured. For this reason, these analyses lasted until seven pm.

The 20th was the last day doing experiments. We did the Glutamine and

Glutamate measurements with the spectrophotometer.

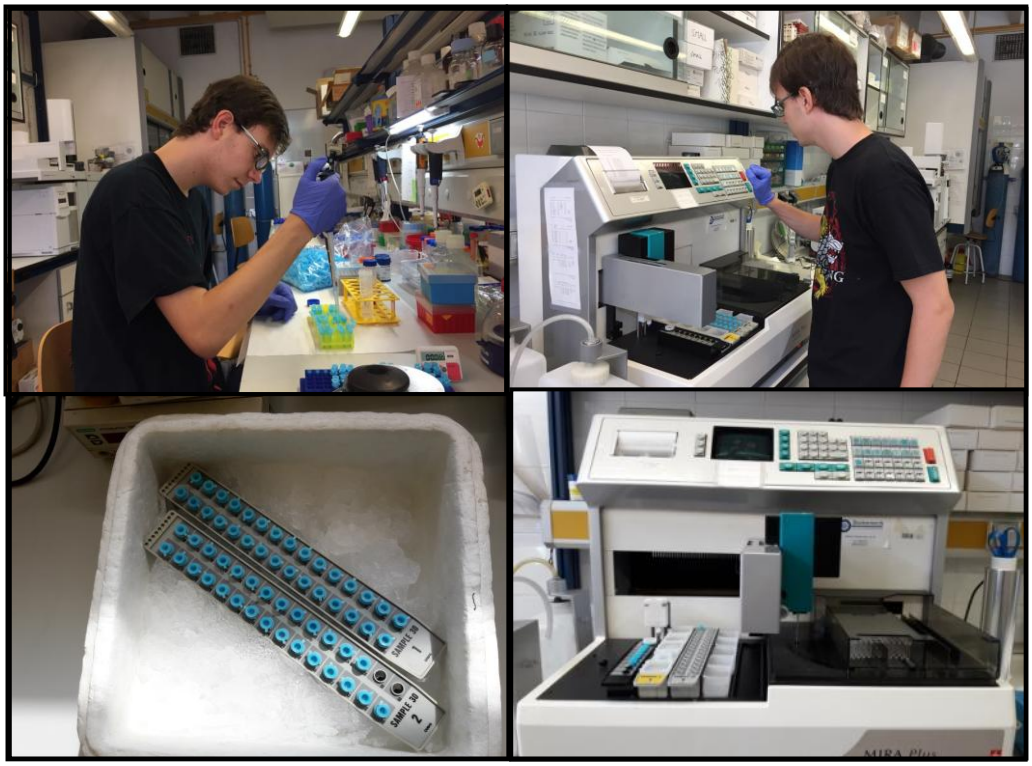
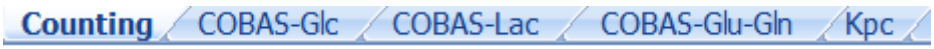


Illustration 52 Pictures of the spectrophotometry analysis week. The pictures from the left show the preparation of the special blue tubes that the COBAS MIRA PLUS uses and the left ones show the machine working and me checking the results.

6.6. *IN VITRO* DATA ANALYSIS

As in any scientific procedure, the information gathered during the experiment must be analysed in order to find a substantial difference between the samples. It may look easy to find if some information is different from another, although the only correct and accepted way to do that in science research is through statistical analytics.

Firstly, I put together all the results from the Scepter and the spectrophotometer and transcribed it to an excel calculation sheet, one for the cell Counting and another for each metabolite analysed.[5]



[5] All the Excel and R Studio calculation sheets and scripts can be found in the annexes VII and VIII

Counting: Many cell samples were diluted to avoid exceeding the maximum value the machine can read, so the first step was multiplying the counting for its dilution. Now that we have the approximated cell value per well, we can proceed to calculate the cell increase in 24 and 48 hours (ΔN).

COBAS: As in the cell samples, many medium samples were also diluted so after calculating an average value between the two or three measurements that we had already done with the COBAS, we multiply it by the dilution.

The objective of arranging this data is to calculate a ratio between the cells and the flux of metabolites (this flux can be calculated for both consumption and production). The way we estimated this ratio is through two formulas:

- Growth tax (μ)
- Production/Consumption Tax (K_{PC})

a)

$$\mu = \frac{\ln\left(\frac{N_f}{N_0}\right)}{t_f}$$

b)

$$k_{PC} = \frac{\Delta M}{\Delta N} \times \mu \times 10^9$$

Equation 6 These are the two formulas used to relate the cell growth with the metabolite flux with proliferative cells, so we assume exponential growth. (**a**) The Growth Tax (μ), where N is the number of cells so N_f is the final cell population, N_0 is the initial population and t_f is the final time, is a correcting factor for the K_{PC} formula taking in account that cell growth is not linear. The units of the Growth Tax are h^{-1}. (**b**) The Production/Consumption Tax (K_{PC}), where ΔN is the increase of cells, ΔM is the increase of metabolite and 10^9 is a conversion factor made of 10^3 to change the mol scale from mmol to μmol and 10^6 is to change from $cell^{-1}$ to $millioncells^{-1}$. The units of the K_{PC} are μmol/million cells*h. [6]

Once this is calculated for every sample both the control cells and the bhb cells, we can start the statistic analysis. The tests I used to analyze these data were Student's Test T, ANOVA, LSD, Duncan, SNK, HSD and Scheffe's method.

Test T student, which I did with excel, is used to compare two experimental groups so I used it to compare the control cells with the bhb ones. It is

[6] These two formulas are inside the useful documents annex IV in the page LI

established to only consider there is a significant variance between data if the result is less than 0.05 (α). (**Table 23** at the annex VII)

The other tests, which I did with RStudio, are used to compare more than two groups, so I used the tests to compare among cell lines with the same condition and all together. But, the ranges among each measurement of the same sample were sometimes too wide so many statistical tests did not found significant differences that may be or not. (**Illustration 58** and **Illustration 59** at annex VIII)

6.7. EXPERIMENTATION *IN SILICO*

After all the in vitro process, I discussed with Carles Foguet, from the computational part of the group, about how to do a computational approach from what I gathered and with the concepts I knew, because learning how to write in programming languages like python would be unbearable within the time frames of this project. During the *Crazy about Biochemistry* I had practised with a free software programme called Optflux which lets you simulated different metabolic related assays like knock outs and flux balances in different environmental conditions or changing the gene expression... from a metabolic model. The one I used[7] in this part of the research is basically a script with the main metabolic pathways from any human cell. This is a Constrained-based model, so we assume steady state.

The experimentation consisted in preparing the data from the experimentation in vitro into an input that allowed the Optflux calculate the flux balance through the different pathways assuming the cell maximizes biomass production (growth). As each cell line had given different information, the inputs will vary so will be able to compare the results of the flux balance simulation and then see if the output qualitatively agrees with the *in vitro* results.

[7] You can find the metabolic model inside a Dropbox on the annex VI

6.7.1. Calculations

The input, which the Optflux needs to do the simulations, is a set of constant metabolites consumption and production by each metabolite the cell can intake. This set is basically the Kpc calculated before and an approximation of a possible Kpc of the other metabolites in the medium that the cell could take profit of. These metabolites are described in the model, but to calculate the Kpc I used the concentrations from the commercial information of the culture's medium.[8]

$$Kpc = -\frac{Metabolite\ Concentration}{\Delta Cell\ number} \cdot \mu \cdot factor$$

Ecuación 7 As we are doing an approximation, the Kpc formula changes a little bit. Firstly we are only calculating the intake to it will always be negative by multiplying the result by -1. Secondly, the factor apart from changing from mmol to µmol and to $cell^{-1}$ to $millioncells^{-1}$ we also multiply it by 0.5 as we assume that they will consume half of the whole metabolite concentration in the medium

As we intend to have global results that we can compare among different conditions and lines doing the Kpc is not enough. After having the Kpc values we must calculate the Kpv which normalizes the data in function of the cell volume.

Ecuación 8 Without calculating the Kpv the biomass production output of the Optflux could not be compared as the consumptions we used as input are in function of the proliferation which depends on the volume. For example if a line proliferates more but has less volume than another line, maybe both had produced an equal quantity of biomass but as the bigger ones must produce more to duplicate themselves the proliferation value will be less and so the Kpc will vary.

$$Kpv = \frac{Kpc}{Cell\ Volume}$$

6.7.2. Simulations

Once we have all the Kpv we can put the values in the metabolic model through a programme called Geany that helps you change the script with your desired values. After programming this input, that will set the boundaries of the consumption/production of external metabolites, we can open the Optflux with this modified model and let the software run a Flux Balance Analysis through a *Wild Simulation*. After, I created environmental conditions which changed values into the ones of the condition to run a simulation of the condition.

[8] DMEM medium composition annex IV page XIX.

Illustration 53 Metabolic model visualized with Geany. This part is the one I used to set the boundaries.

To sum up, the biomass productions were:

	SW480	SW620	SW620-LiM2
Control	7.848511E-6	NaN	Kpc: 9.2178541
Presence of bhb	1.1128035E-5	1.0291501E-5	Kpc: 9.6605046

Table 6 Summary of the biomass production from each simulation. The SW620-LiM2 results were done with Kpc instead of Kpv as both control and condition had done NaN and they could not be compared.[9]

[9] Optflux pictures of the whole simulation at the annex IX.

7. CONCLUSIONS

At the end of any scientific experimentation it is time to discuss the results and develop conclusions to show that all the time spent on the project has a result. These might agree with your hypothesis, or they might reject it. The conclusions I reached to are classified in three groups: Experimental (*in silico* and *in vitro*), Theoretical and Personal.

- *In vitro* Experimental Conclusions

After the statistical analysis of the entire data gathered during the *in vitro* experimentation, at first I would reach the following conclusion: “As most of the differences found are not meaningful; neither the metabolism flux, nor the proliferation have been affected by the condition”. But if we look at the data closely, we can see that the 48h Control proliferation standard deviation (σ) between the replicates of the cell lines is above 5, therefore it affects the statistical tests used between the control and the condition samples, and it must affect the Kpc values as they are in function of the proliferation.

Therefore, we should repeat the experiment to ensure if those results were not enough accurate and discover if the β-hydroxybutyrate (bhb) had any effect on their metabolism. However, we cannot do that as experimentation is expensive and this project could not last more than the time I gratefully spent at the UB, so I will discuss the results assuming that at 48h the lines had a meaningful increase or decrease of proliferation both in control and in the presence of bhb, as the only value of their growth was the mean between the three replicates.

At 24h the cell growths are pretty similar and it is not enough time to get big effects, so we could say that the first statement of “it had no effects” could be applied, except for SW480 and SW620. SW480 had no meaningful difference in proliferation, but the lines with the bhb had consumed less glucose and produced much more glutamate. This would mean that the cells in bhb had turned it into acetyl-CoA and then into glutamate through the Krebs cycle that

would be accumulated and exported in the medium afterwards. SW620, instead, had a meaningful difference in proliferation and also in those two metabolites. The control had proliferated more than the condition and they had also consumed more glucose and exported less glutamate which might had gone to produce amino acids and nucleotides what would explain why the control had proliferated a bit more. Moreover, the lactate production is really similar in both, and as the consumption of glucose in the control is the double of the one with the condition, they probably took profit of the bhb in the medium to turn it into Acetyl-CoA and then produce lactate turning it into pyruvate.

At 48h if we do the previously mentioned assumption, all the controls had proliferated differently from the conditions. In sw480 and SW620 the control had proliferated more, but in SW620-LiM2 the condition is the one that had grown more. In SW480 and SW620 we could explain the control proliferation as the conditions with bhb tend to accumulate glutamate that would not be used to grow and we also could say that the condition cells have profited by the bhb as the Glc consumption is less than in the control but the lactate productions are pretty similar. Moreover, we can notice that SW620 is the only line that Glc consumption is so similar. Finally, we could say that in SW620-LiM2 the condition had grown more as they took profit of the bhb.

- *In silico* Experimentation Conclusions

Comparing the results within the same lines we find that the model says that the three conditions have produced more biomass that without it. What if we look at the proliferation from *in vitro* we know it only happens with SW620-LiM2. Therefore, I can say that or this model is not enough accurate for the lines, or the deviation problems from in vitro had been brought here.

But as we assumed there was no variation, then the problems would be on the cell model. These could be many different things from not taking in account the Warburg effect to assume they always maximize biomass production, or even the steady state assumption when the model was build. Or maybe as the models do not have all the know reactions, we are missing a pathway. The possibilities are infinite. But if we repeated or gathered more data to build a

model that could accomplish good simulations the amount of experiments we could do in a few time with some computer programmes would be massive.

- Theoretical Conclusions

Life is incredibly complex, as we have seen on the three first parts (Basics of Biology and Chemistry, Metabolism and Cancer) through the multiple explanations about the building blocks of life (biomolecules) how they are synthesized (metabolism) and also how the cell regulate many functions to decide what to give priority to through the signalling pathways that in cancer are deregulated due to oncogenes. That complexity let us comprehend why life sciences are tending to work through systemic approaches and computer modelling to find results working with all this networks as a whole.

- Personal Conclusions

This whole project is the biggest work I have ever achieved. I have been able to learn and comprehend subjects far from my understanding before starting it which also helped me to decide how I would like to lead my future career. The experience in the university's laboratories had been educational in many senses, from all the concepts summarized along this memory to the understanding of the importance of organizing and taking responsibilities when working in scientific research.

8. SOURCES

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Joan J. Guinovart speech about Diabetes at *"I tu? Jo, Bioquímica"*

Colorectal cancer projects explanation during a visit to the IRB at the open day.

Centre de Regulació genòmica (CRG) speech at the PRBB's open day.

Mathematical models speech by Dr. Mircea Sofonea at the 2nd *Science Needs You.*

8.5. FIGURES' SOURCES

Illustrations

Illustration 1 < Own elaboration >

Illustration 2 < https://the-history-of-the-atom.wikispaces.com/Niels+Bohr >

Illustration 3 < https://sciencenotes.org/printable-color-periodic-table-2017/ >

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Illustration 11 < https://www.ck12.org/biology/Exocytosis-and-Endocytosis/lesson/Exocytosis-and-Endocytosis-BIO/ >

Illustration 12 < https://pdb101.rcsb.org/learn/videos/how-enzymes-work >

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Illustration 14 < http://biology.tutorpace.com/ribosomes-online-tutoring >

Illustration 15 < http://www.4to40.com/science/print.asp?p=Rough-Endoplasmic_Reticulum_Function >

Illustration 16 < https://en.wikipedia.org/wiki/Golgi_apparatus >

Illustration 17 < https://micro.magnet.fsu.edu/cells/lysosomes/lysosomes.html >

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Illustration 20 < https://www.saddlespace.org/whittakerm/science/cms-page/view/7795235 >

Illustration 21 < https://en.wikipedia.org/wiki/Nicotinamide_adenine_dinucleotide https://en.wikipedia.org/wiki/Nicotinamide_adenine_dinucleotide_phosphate https://en.wikipedia.org/wiki/Flavin_adenine_dinucleotide >

Illustration 22 < https://en.wikipedia.org/wiki/Adenosine_Tri-Phosphate_(band) https://en.wikipedia.org/wiki/Coenzyme_A >

Illustration 23 [13]

Illustration 24 [13]

Illustration 25 [14]

Illustration 26 < https://www.quora.com/Where-does-the-hydrogen-go-during-glycolysis >

Illustration 27 [14]

Illustration 28 [14]

Illustration 29 < https://www.shmoop.com/cell-respiration/oxidative-

phosphorylation.html >

Illustration 30 [14]

Illustration 31 [14]

Illustration 32 [14]

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Illustration 43 < http://ferretronix.com/march/computer_cards/sdcard/ >

Illustration 44 < http://www.genome.jp/kegg/pathway/map/map01100.html >

Illustration 45 < https://getrevising.co.uk/revision-cards/motion-22 >

Illustration 46 Made out of notes from the *Bioinformatic approach to biological questions*

Illustration 47 [16]

Illustration 48 Pictures took by me.

Illustration 49 Pictures took by me.

Illustration 50 Pictures took by me.

Illustration 51 Pictures took by me.

Illustration 52 Pictures took by me.

Illustration 53 Screenshot took by me.

Illustration 54 < https://chem.libretexts.org/Core/Physical_and_Theoretical_Chemistry/Kinetics/Reaction_Rates/Experimental_Determination_of_Kinetcs/Spectrophotometry >

Illustration 55 Picture took by me

Illustration 56 Picture took by me

Illustration 57 Screenshot took by me.

Illustration 58 Screenshots took by me.

Illustration 59 Screenshot took by me.

Equations are from the annex Useful documents or they were elaborated by me.
Diagrams and **Tables** were all elaborated by me.

Annexes

ANNEX I: Glossary

Acetyl-CoA: the acetylated form of coenzyme A, formed as an intermediate in the oxidation of carbohydrates, fats, and protein in animal metabolism.

Adenosine triphosphate (ATP): an ester of adenosine and triphosphoric acid, $C_{10}H_{12}N_5O_4H_4P_3O_9$, formed especially aerobically by the reaction of ADP and an orthophosphate during oxidation, or by the interaction of ADP and phosphocreatine or certain other substrates, and serving as a source of energy for physiological reactions, especially muscle contraction.

Aerobic: (of an organism or tissue) requiring the presence of air or free oxygenfor life.

Aldose: a sugar containing the aldehyde group or its hemiacetal equivalent.

Alpha helix: the rod like spatial configuration of many protein molecules in which the polypeptide backbone is stabilized by hydrogen bonds between amino acids in successive helical turns.

Amine: any of a class of compounds derived from ammonia by replacement ofone or more hydrogen atoms with organic groups.

Anaplasia: the loss of structural differentiation within a cell or group of cells.

Angiogenesis: the formation and development of blood vessels.

Antibodies: any of numerous Y shaped protein molecules produced by B cells as a primary immune defence, each molecule and its clones having a unique binding site that can combine with the complementary site of a foreign antigen, as on a virus or bacterium, thereby disabling the antigen and signalling other immune defences.

Apoptosis: a genetically regulated process leading to the death of cells and triggered by the presence or absence of certain stimuli, as DNA damage.

Autophagy: controlled digestion of damaged organelles within a cell.

Biochemistry: the science dealing with the chemistry of living matter.

Bioelement: Any chemical element that is found in the molecules and compounds which make up a living organism. In the human body the most common bioelements (in decreasing order of occurrence) are oxygen, carbon, hydrogen, nitrogen, calcium, and phosphorus. Other bioelements include sodium, potassium...

Carbohydrates: any of a class of organic compounds that are polyhydroxy aldehydes or polyhydroxy ketones, or change to such substances on simple chemical transformations, as hydrolysis, oxidation, or reduction, and that form the supporting tissues of plants and are important food for animals and people.

Carboxylic acid: any organic acid containing one or more carboxyl groups.

Catalysts: a substance that causes or accelerates a chemical reaction without itself being affected.

Cell Lines: also known as immortalised cell lines, is a population of cells from a multicellular organism which would normally not proliferate indefinitely but, due to mutation, have evaded normal cellular senescence and instead can keep undergoing division. The cells can therefore be grown for prolonged periods *in vitro*. The mutations required for immortality can occur naturally or be intentionally induced for experimental purposes. Immortal cell lines are a very important tool for research into the biochemistry and cell biology of multicellular organisms. Immortalised cell lines have also found uses in biotechnology.

Cellulose: an inert carbohydrate, $(C_6H_{10}O_5)_n$, the chief constituent of the cell walls of plants and of wood, cotton, hemp, paper, etc.

Chemotherapy: the treatment of disease by means of chemicals that have a specific toxic effect upon the disease-producing microorganisms or that selectively destroy cancerous tissue.

Chloroplasts: a plastid containing chlorophyll.

Cytoskeleton: a shifting lattice arrangement of structural and contractile components distributed throughout the cell cytoplasm, composed of microtubules, microfilaments, and larger filaments, functioning as a structural support and transport mechanism.

Cytosol: the water-soluble components of cell cytoplasm, constituting the fluid portion that remains after removal of the organelles and other intracellular structures.

Cytotoxin: a substance that has a toxic effect on certain cells.

Disaccharides: any of a group of carbohydrates, as sucrose or lactose, that yield monosaccharides on hydrolysis.

DNA: deoxyribonucleic acid: an extremely long macromolecule that is the main component of chromosomes and is the material that transfers genetic characteristics in all life forms, constructed of two nucleotide strands coiled around each other in a ladder like arrangement with the sidepieces composed of alternating phosphate and deoxyribose units and the rungs composed of the purine and pyrimidine bases adenine, guanine, cytosine, and thymine: the genetic information of DNA is encoded in the sequence of the bases and is transcribed as the strands unwind and replicate.

Endothermic: noting or pertaining to a chemical change that is accompanied by absorption of heat (opposed to exothermic).

Eukaryote: any organism having as its fundamental structural unit a cell type that contains specialized organelles in the cytoplasm, a membrane-bound nucleus enclosing genetic material organized into chromosomes, and an elaborate system of division by mitosis or meiosis, characteristic of all life forms except bacteria, blue-green algae, and other primitive microorganisms.

Exothermic: noting or pertaining to a chemical change that is accompanied by aliberation of heat (opposed to endothermic).

Fermentation: is a metabolic process that consumes sugar in the absence of oxygen.

Flavin adenine dinucleotide (FAD): is a redox cofactor, more specifically a prosthetic group of a protein, involved in several important enzymatic reactions in metabolism.

Fructose: a yellowish to white, crystalline, water-soluble, levorotatory ketose sugar, $C_6H_{12}O_6$, sweeter than sucrose, occurring in invert sugar, honey, and a great many fruits: used in food stuffs and in medicine chiefly in solution as an intravenous nutrient.

Furanose: A sugar having a cyclic structure resembling that of furan.

Genomics: the study of the whole group of genes, the genome.

Glucose (Glc): a sugar, $C_6H_{12}O_6$, having several optically different forms, the common dextrorotatory form (dextroglucose, or -glucose) occurring in many fruits, animal tissues and fluids, etc., and having a sweetness about one half that of ordinary sugar, and the rare levorotatory form (levoglucose, or -glucose) not naturally occurring.

Glutamate (Glu): a salt or ester of glutamic acid.

Glutamine (Gln): a crystalline amino acid, $HOOCCH(NH_2)CH_2CH_2CONH_2$, related toglutamic acid.

Glycerol: a colorless, odorless, syrupy, sweet liquid, $C_3H_8O_3$**,** usuallyobtained by the saponification of natural fats and oils: used for sweetening and preserving food, in the manufacture of cosmetics,perfumes, inks, and certain glues and cements, as a solvent andautomobile antifreeze, and in medicine in suppositories and skinemollients.

Glycogen: a white, tasteless polysaccharide, $(C_6H_{10}O_5)_n$**,** molecularly similarto starch, constituting the principal carbohydrate storage material inanimals and occurring chiefly in the liver, in muscle, and in fungi andyeasts.

Glycosidic bond: is a type of covalent bond that joins a carbohydrate (sugar) molecule to another group, which may or may not be another carbohydrate.

Glycosphingolipid: Is a subtype of glycolipids containing the amino alcohol sphingosine.

Hexose: any of a class of sugars containing six atoms of carbon, including glucose and fructose.

hnRNA: heterogeneous nuclear RNA

Homeostatic: the tendency of a system, especially the physiological system of higher animals, to maintain internal stability, owing to the coordinated response of its parts to any situation or stimulus that would tend to disturb its normal condition or function.

Hydrolase: an enzyme that catalyzes hydrolysis.

iRNA: interference RNA

Ketoses: monosaccharides that contains a ketone group.

Kinase: a transferase that catalyzes the phosphorylation of a substrate by ATP.

Lactate (Lac): an ester or salt of lactic acid.

Lactose: a disaccharide, $C_{12}H_{22}O_{11}$, present in milk, that upon hydrolysis yields glucose and galactose.

Maltose: a white, crystalline, water-soluble sugar, $C_{12}H_{22}O_{11}H_2O$, formed by the action of diastase, especially from malt, on starch: used chiefly as a nutrient, as a sweetener, and in culture media.

Metabolomics: the study of all the metabolites present in cells, tissues, and organs

Metastasis: the transference of disease-producing organisms or of malignant or cancerous cells to other parts of the body by way of the blood orlymphatic vessels or membranous surfaces.

miRNA: micro RNA

Monomer: a molecule of low molecular weight capable of reacting with identical or different molecules of low molecular weight to form a polymer.

Monosaccharide: a carbohydrate that does not hydrolyze, as glucose, fructose, or ribose, occurring naturally or obtained by the hydrolysis of glycosides or polysaccharides.

mRNA: messenger RNA

Mutation: a sudden departure from the parent type in one or more heritable characteristics, caused by a change in a gene or a chromosome.

NAD: nicotinamide adenine dinucleotide: a coenzyme, $C_{21}H_{27}N_7O_{14}P_2$, involved in many cellular oxidation-reduction reactions.

NADP: nicotinamide adenine dinucleotide phosphate: a coenzyme, $C_{21}H_{28}N_7O_{17}P_3$, similar in function to NAD in many oxidation-reduction reactions.

Necrosis: death of a circumscribed portion of animal or plant tissue.

Neoplastic: a new, often uncontrolled growth of abnormal tissue tumour.

Nitrogenous base: a nitrogen-containing organic compound that has the chemical properties of a base, especially a pyrimidine or purine.

Non-saponifiable: it cannot undergo the saponification reaction.

P53: A protein that is thought to play a role in regulating cell death or apoptosis, in suppressing tumours, in regulating the cell cycle, and in stopping the cell from dividing when the DNA is damaged.

Pentose: a monosaccharide containing five atoms of carbon.

Peptide: a compound containing two or more amino acids in which the carboxyl group of one acid is linked to the amino group of the other.

Ph: the symbol for the logarithm of the reciprocal of hydrogen ion concentration in gram atoms per litre, used to express the acidity or alkalinity of a solution on a scale of 0 to 14, where less than 7represents acidity, 7 neutrality, and more than 7 alkalinity.

Phenotype: the appearance of an organism resulting from the interaction of the genotype and the environment.

Phosphosphingolipids: molecules made of the combination of a phosphate *with a* sphingolipid.

Polymer: a compound of high molecular weight derived either by the addition of many smaller molecules, as polyethylene, or by the condensation of many smaller molecules with the elimination of water, alcohol, or the like, as nylon.

Polysaccharide: a carbohydrate, as starch, glycogen, or cellulose, containing more than three monosaccharide units per molecule, the units being attached to each other.

Prokaryote: any cellular organism that has no nuclear membrane, no organelles in the cytoplasm except ribosomes, and has its genetic material in the form of single continuous strands forming coils or loops, characteristic of all organisms in the kingdom Monera, as the bacteria and blue-green algae.

Prostaglandin: any of a class of unsaturated fatty acids that are involved in the contraction of smooth muscle, the control of inflammation and body temperature, and many other physiological functions.

Proteomics: the study of the functions, structures, and interactions of proteins; the study of the proteome.

Pyranose: any monosaccharide having a pyran ring structure.

Pyrrolysine: is an α-amino acid that is used in the biosynthesis of proteins in some Archaea and Bacteria. It contains an α-amino group (which is in the protonated $-^{+}NH_3$ form under biological conditions), a carboxylic acid group (which is in the deprotonated $-COO^{-}$ form under biological conditions). Its pyrroline side-chain is similar to that of lysine in being basic and positively charged at neutral pH.

Pyruvate: an ester or salt of pyruvic acid.

Retinoblastoma proteins (pRB): is a tumour suppressor protein that is dysfunctional in several major cancers

Ribose: a white, crystalline, water-soluble, slightly sweet solid, $C_5H_{10}O_5$, a pentose sugar obtained by the hydrolysis of RNA.

RNA: ribonucleic acid: any of a class of single-stranded molecules transcribed from DNA in the cell nucleus or in the mitochondrion or chloroplast, containing along the strand a linear sequence of nucleotide bases that is complementary to the DNA strand from which it is transcribed: the composition of the RNA molecule is identical with that of DNA except for the substitution of the sugar ribose for deoxyribose and the substitution of the nucleotide base uracil for thymine.

Saponify: to decompose (any ester), forming the corresponding alcohol and acid or salt.

Saturated: (of a solution) containing the maximum amount of solute capable of being dissolved under given conditions.

Spectrophotometer: an instrument for making photometric comparisons between parts of spectra.

Steroid: any of a large group of fat-soluble organic compounds, as the sterols, bile acids, and sex hormones, most of which have specific physiological action.

Stoichiometry: the calculation of the quantities of chemical elements or compounds involved in chemical reactions.

Sucrose: a crystalline disaccharide, $C_{12}H_{22}O_{11}$, the sugar obtained from the sugarcane, the sugar beet, and sorghum, and forming the greater part of maple sugar

Terpene: this class or any of its oxygenated derivatives, any hydrocarbon from the same source having the formula C_5H_8 (hemiterpene) the formula $C_{10}H_{16}$ with an aliphatic structure (acyclic terpene) or two-ringed structure (bicyclic terpene) the formula $C_{15}H_{24}$ (sesquiterpene) etc., and any of their oxygenated derivatives.

Thiol: any of a class of sulphur-containing organic compounds with the formula R-SH, where R is an organic group.

T-Lymphocytes: any of several closely related lymphocytes, developed in the thymus, that circulate in the blood and lymph and orchestrate the immune system's response to infected or malignant cells, either by lymphocyte secretions or by direct contact: helper T cells recognize foreign antigen on the surfaces of other cells, then they stimulate B-cells to produce antibody and signal killer T cells to destroy the antigen-displaying cells; subsequently suppressor T cells return the immune system to normal by inactivating the B cells and killer T cells.

Unsaturated: not saturated; having the power to dissolve still more of a substance.

β-hydroxybutyrate: also known as β-Hydroxybutyric acid and 3-hydroxybutyric acid, is an organic compound and a beta hydroxy acid with the formula $CH_3CH(OH)CH_2CO_2H$; its conjugate base is *beta*-hydroxybutyrate, also known as 3-hydroxybutyrate.

ANNEX II: Methodology

In the in vitro part of my experimentation there are two machines that gave me the most quantity of data:

- Spectrophotometer
- Scepter Cell Counter

But, these machines would be meaningless without the exhaustive statistical analysis behind it which is explained afterwards.

SPECTROPHOTOMETER

Human beings enjoy chromatic sight, which means that we see the colour of the things around us. But which are the differences that make some objects red, green or blue? We say an object is red, for example, when it absorbs all the white light frequencies but the red one, which is reflected out and then you can see it.

Now, imagine a Chinese shadows show. You can't see the person or people that are producing those images, but what you can see is the shadow produced by the light the absorbed.

Basically a spectrophotometer is a machine we use to calculate the light absorbance from a substance compared to another with null concentration.

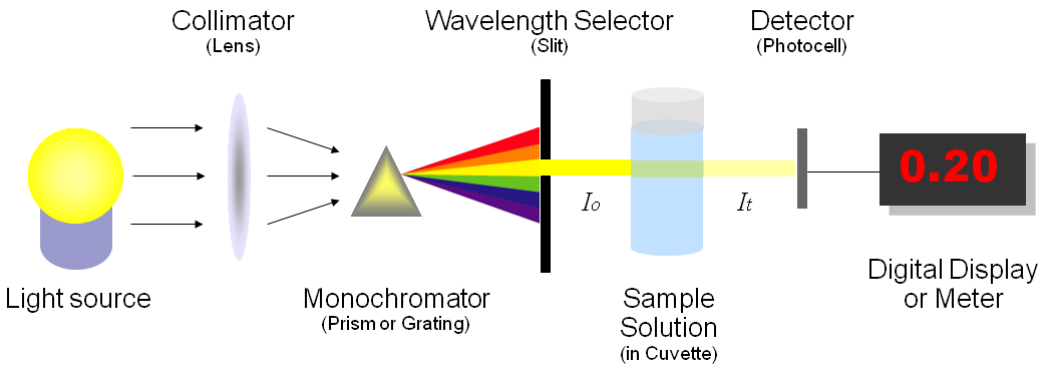

Illustration 54 is a graphical representation of the different engines that build the spectrophotometer. A light source that is changed to only one wave length that will pass through a cuvette with our sample and then a light detector will calculated the difference amount of light had reach through due to the dissolved molecules.

We get nothing from getting a relative absorbance if we leave it like that, but if we do a linear regression with the concentration of the samples we could then discover the approximate concentration of an unknown sample if we measured its absorbance. The formula that is used to express the standard line is the Lambert Beer Law:

$$A = \varepsilon\, l\, c$$

Equation 9 This equation is the Lambert Beer law. The Absorbance (A) equals the product of the Molar absorptivity (ε) by the cuvettes length (l) by the concentration of the solution (c).This equation is used to do a standard line with which we can find any unknown concentration from its absorbance.

The commercial name of the spectrophotometer we used is COBAS Mira Plus.

Illustration 55 is a picture of the COBAS Mira Plus I took while it was cleaning its needles.

SCEPTER CELL COUNTER

Nowadays there are some different methods to count the cells on a culture, for example the classic Neubauer chamber in which you had to count them manually and mathematically approach to the number in your solution. The Scepter Cell Counter is a machine that automatically counts cells up to $5*10^5$ cells/mL. It is based on the Coulter principle of impedance-based particle detection.

The Coulter principle states that particles pulled through an orifice, concurrent with an electric current, produce a change in impedance that is proportional to the volume of the particle traversing the orifice. This pulse in impedance originates from the displacement of electrolyte caused by the particle.

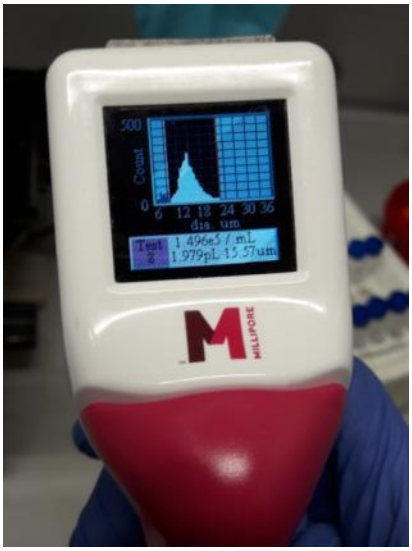

Illustration 56 is a close image of the Scepter screen after completing a measurement. This pipette like engine tells you the density of cells and its approximated volume in pL.

STATISTICS MEASURES AND TESTS

An explanation of every statistic calculation used in order to manage the data with the Excel and the RStudio.

Average: arithmetic mean.

Standard Deviation (σ): a measure of dispersion in a frequency distribution, equal to the square root of the mean of the squares of the deviations from the arithmetic mean of the distribution.

Standard lining: a system for aligning type so that all fonts of the same point size have a common baseline.

Student's t-test: is any statistical hypothesis test in which the test statistic follows a Student's t-distribution under the null hypothesis.

ANOVA: Analysis of variance (ANOVA) is a collection of statistical models used to analyze the differences among group means and their associated procedures (such as "variation" among and between groups), developed by statistician and evolutionary biologist Ronald Fisher.

Duncan’s test: In statistics, Duncan's new multiple range test (MRT) is a multiple comparison procedure developed by David B. Duncan in 1955.

Duncan's MRT belongs to the general class of multiple comparison procedures that use the studentized range statistic qr to compare sets of means.

Scheffé's method: is a method for adjusting significance levels in a linear regression analysis to account for multiple comparisons. It is particularly useful in analysis of variance (a special case of regression analysis), and in constructing simultaneous confidence bands for regressions involving basis functions.

Student–Newman–Keuls test (SNK): method is a stepwise multiple comparisons procedure used to identify sample means that are significantly different from each other. It was named after Student (1927), D. Newman, and M. Keuls. This procedure is often used as a post-hoc test whenever a significant difference between three or more sample means has been revealed by an analysis of variance (ANOVA).

Tukey's HSD (honest significant difference) test: or the Tukey–Kramer method, is a single-step multiple comparison procedure and statistical test. It can be used on raw data or in conjunction with an ANOVA (post-hoc analysis) to find means that are significantly different from each other. Named after John Tukey, it compares all possible pairs of means, and is based on a studentized range distribution (q) (this distribution is similar to the distribution of t from the t-test. See below). The Tukey HSD tests should not be confused with the Tukey Mean Difference tests (also known as the Bland–Altman diagram).

Least Significant Difference test (LSD): When you run an ANOVA (Analysis of Variance) test and get a significant result, that means at least one of the groups tested differs from the other groups. However, you can’t tell from the ANOVA test which group differs. In order to address this, Fisher developed the least significant difference test in 1935, which is only used when you reject the null hypothesis as a result of your hypothesis test results. The LSD calculates the smallest significant between two means as if a test had been run on those two means (as opposed to all of the groups together). This enables you to make direct comparisons between two means from two individual groups. Any difference larger than the LSD is considered a significant result.

ANNEX III: First Week Protocols

These are two protocols about the first week experimentation explaining all the material, machines and procedures I used. These two are written by me after taking notes of the experimentation process.

Part 1: Cell culture (in a laminar flux Hood)

Objective

Seed a cell line in a culture from a previous petri dish (or 100mm plate). You must seed some to keep the line for future studies in a 100mm plate and some in the 6 wells plate to study them.

Material

- Cells: SW480, SW620 and SW620-LiM2 (previously warmed and cultured)
- Culture medium(Look at page X to see the composition)
- PBS (Phosphate Buffered Saline)
- Trypsin
- 100 mm and 6 well culture plate
- β-hydroxybutiric acid sodium salt (bhb)
- H2O milli Q sterile
- 50mm and 15mmfalcons
- 5ml, 10ml and 25ml pipettes
- Glass Pasteur pipettes
- Automatic pipette pump
- 200µl and 1ml micropipette
- Micropipette's tips
- Scepter cell counter and its tips
- Ethanol
- Void system
- Detergent
- Autoclave
- Coat and nitrile gloves (L size)
- Paper Tissue
- Flux Hood

Procedure

- Put on your gloves and coat

- Warm up the medium, the trypsin, and the PBS in bain-marie during 15' until it gets 37°C
- Sterilize all the tools that will be used in the hood using some paper tissues soaked in ethanol or autoclave.
- Switch off the UV light and turn on the white light and the fan inside the hood.
- Wash your gloves with ethanol to put out the cultures from the incubator avoiding chances of contaminating neither your cultures nor the others inside.
- Check the cells in the microscope (40x)
- Take one or two 100mm plates depending on how much cells you see and put them inside the hood.
- Suck the medium with the void system (with a glass Pasteur pipette on the edge) and add 5ml of PBS on the culture intending to clean it.
- Change the PBS for 2ml of trypsin and incubate the plates for 5 minutes.
- Put out the plates from the incubator and add 3 times more medium than trypsin (6ml) and collect everything in the plate inside a Corning.
- Centrifuge the falcons between 500g and 1500g (depending on the cell line)during 5' to divide their content in two parts: the pellet (cells) and the supernatant (medium, trypsin, etc.).
- We bring everything back to the hood and suck up the supernatant with the void system and add 2ml of fresh medium and resuspend the pellet using the same pipette.
- To count how many cells we have (approximately), we prepare a dilution 1/20 with 950µl of PBS and 50 from the falcons in an eppendorf, one for each falcon, so one for each cell line.
- Use a Scepter counter with its tips to count automatically how many cells we have so we can calculate how much of the falcon's solution must be added to a 30ml solution so we seed 150.000 cells.
- Prepare a falcon per cell line with 30ml of fresh medium using a 25ml pipette.
- Add the µl of falcons' solution got in the calculation and mix it to get a homogeneous solution of cells.
- Label two 100mm plates for each cell line and some 6 wells plates, in that case 7.
- Seed 2ml of falcon's solution in each well in the 6 wells plate and 10ml of fresh medium and 200µl of falcon's solution in the 100mm so we can store line for future experiments.
- Incubate the plates between 6 to 24 hours to let the cells add on the plate's surface.
- Once they are added to the plate you can add the medium of study.
- Some of the wells are labelled as Control; those will get 3ml of fresh medium. The others, labelled as bhb, will get 3ml of medium with 5mM of β-hydroxybutyrate (as it is in water it is dissociated from sodium).

- Keep some of both mediums for further analysis in a 15mm falcon each and freeze them.
- Clean your work zone: the surface of the hood with a paper tissue soaked in ethanol, the void system sucking some detergent and throw away the material you can't reuse (pipettes, micropipette's tips, used plates and falcons…)

Part2: Gather some data

Objective

Count and take pictures of the cells and keep their medium for further analysis. You will have to do this three times (for the 0hours, 24hours and 48 hours samples) with little variations depending on the samples.

Material

- Cultured Cells(SW480, SW620 and SW620-LiM2) in a 6 wells plate
- PBS (Phosphate Buffered Saline)
- Trypsin
- 15mm cornings/falcons
- 5ml, 10ml and 25ml pipettes
- Glass Pasteur pipette
- Automatic pipette pump
- 200µl and 1ml micropipette
- Micropipette's tips
- Scepter cell counter
- Ethanol
- Void system
- Detergent
- Autoclave
- Coat and gloves
- Paper Tissue
- Flux Hood
- Microscope
- Computer
- Microscope camera gadget

Procedure

- Put on your gloves and coat
- Warm up the medium, the trypsin, and the PBS in bain-marie during 15' until it gets 37°C
- Sterilize all the tools that will be used in the hood using some paper tissues soaked in ethanol or the autoclave.

- Switch off the UV light and turn on the white light and the fan inside the hood.
- Wash your gloves with ethanol to put out the cultures from the incubator avoiding chances of contaminating neither your cultures nor the others inside.
- Switch on the computer and attach the gadget connected to the microscope to it.
- Take some pictures of the cells at 40x and 100x and save them as .tif.
- Sterilize all the tools that will be used inside the hood using some paper tissues soaked in ethanol or the autoclave.
- Switch off the UV light and turn on the white light and the fan inside the hood.
- Put the material inside the hood.
- Gather the medium from well and keep each one in a falcon and freeze them. If you are doing the 0h culture you don't need to collect it, you can just suck it apart, as you have kept it while preparing the culture.
- Now add PBS (1ml) and suck it, then add trypsin (400µl) and then add 3 times more medium than trypsin (1.2ml) and store everything in a 15ml falcon, repeat that with every well from the plate.
- Centrifuge them all at 1500g during 5 minutes at 37°C.
- Suck apart the supernatant and resuspend the pellet in 1ml of PBS.
- Prepare eppendorfs (1 for each falcon) with 500µl of solution (or a dilution from that one if the machine is unable to count the high amount of cells)
- Use the Scepter with its tips to count how many cells there's in the solution and to calculate its volume.
- Collect the results for future analysis.

Part 3: Spectrophotometry analysis

Objective

Find out the concentrations of Glucose (Glc), Lactate (Lac), Glutamine (Gln) and Glutamate (Glu) from the medium samples with the spectrophotometer.

Material

- Reagents
- Samples
- COBAS Mira Plus and its add-ons

Procedure

COBAS MIRA PLUS protocols in the next annex page XXXIX.

ANNEX IV: Useful Documents

In this chapter you will find some documents that I gathered in order to complete this project.

- DMEM medium information
- B-hydroxybutyrate commercial information
- Culture room rules and plates surface area
- COBAS MIRA PLUS protocol
- Glucose, Lactate, Glutamate and Glutamine information to analyze with COBAS
- Kpc formulas

Technical Resources - Media Formulations

11966 - DMEM, no glucose

Catalog Number(s)

11966025

Components	Molecular Weight	Concentration (mg/L)	mM
Amino Acids			
Glycine	75.0	30.0	0.4
L-Arginine hydrochloride	211.0	84.0	0.39810428
L-Cystine 2HCl	313.0	63.0	0.20127796
L-Glutamine	146.0	584.0	4.0
L-Histidine hydrochloride-H2O	210.0	42.0	0.2
L-Isoleucine	131.0	105.0	0.8015267
L-Leucine	131.0	105.0	0.8015267
L-Lysine hydrochloride	183.0	146.0	0.7978142
L-Methionine	149.0	30.0	0.20134228
L-Phenylalanine	165.0	66.0	0.4
L-Serine	105.0	42.0	0.4
L-Threonine	119.0	95.0	0.79831934
L-Tryptophan	204.0	16.0	0.078431375
L-Tyrosine disodium salt dihydrate	261.0	104.0	0.39846742
L-Valine	117.0	94.0	0.8034188
Vitamins			
Choline chloride	140.0	4.0	0.028571429
D-Calcium pantothenate	477.0	4.0	0.008385744
Folic Acid	441.0	4.0	0.009070295
Niacinamide	122.0	4.0	0.032786883
Pyridoxine hydrochloride	206.0	4.0	0.019417476
Riboflavin	376.0	0.4	0.0010638298

Components	Molecular Weight	Concentration (mg/L)	mM
Thiamine hydrochloride	337.0	4.0	0.011869436
i-Inositol	180.0	7.2	0.04
Inorganic Salts			
Calcium Chloride (CaCl2) (anhyd.)	111.0	200.0	1.8018018
Ferric Nitrate (Fe(NO3)3"9H2O)	404.0	0.1	2.4752476E-4
Magnesium Sulfate (MgSO4) (anhyd.)	120.0	97.67	0.8139166
Potassium Chloride (KCl)	75.0	400.0	5.3333335
Sodium Bicarbonate (NaHCO3)	84.0	3700.0	44.04762
Sodium Chloride (NaCl)	58.0	6400.0	110.344826
Sodium Phosphate monobasic (NaH2PO4-H2O)	138.0	125.0	0.9057971
Other Components			
Phenol Red	376.4	15.0	0.039851222

Referencia

1. Dulbecco, R. and Freeman, G. (1959) Virology 8:396.

In addition to these concentrations, we also added:

- 12,5mM of Glucose
- 5% of Fetal Bovine Serum (FBS)
- 1% Streptomycin/ Penicillin

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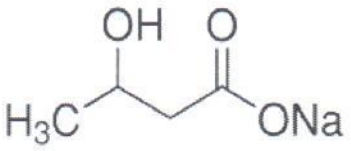
Product Specification

Product Name:
DL-β-Hydroxybutyric acid sodium salt - ~98%

Product Number:	**H6501**
CAS Number:	150-83-4
MDL:	MFCD00016716
Formula:	C4H7NaO3
Formula Weight:	126.09 g/mol
Storage Temperature:	2 - 8 °C



TEST	Specification
Appearance (Color)	White
Appearance (Form)	Powder
Solubility (Turbidity)	Clear
Solubility (Color)	Colorless
1g in 10ml of Water	
Proton NMR spectra	Conforms to Structure
Water (by Karl Fischer)	≤ 1.0 %
Purity (Titration by HCL04)	97.5 - 102.5 %
Recommended Retest Period	----------
6 Years	

Specification: PRD.0.ZQ5.10000020801

CULTIVOS CELULARES

INSTRUCCIONES

Belén Alvarez Palomo

INDICE:

-Contaminación
    -Bacterias
    -Hongos
    -Levaduras
    -Micoplasma

-Normas de esterilidad

-El incubador
    -Elementos a controlar
    -Limpieza

-La campana

-La bomba

-Pedidos

-Material del laboratorio

-Casas comerciales

-Bibliografía consulta

-Anexos:

1- Modelo hoja de registro celular
2- Capítulo 20 (Culture de cellules musculaires squeletiques) de *Culture de Cellules Animales*. M. Adolphe, G. Barlovatz-Meimon.
3- Notas sobre cultivos del catálogo de BioWhittaker-Cultivos Celulares
4- Principales protocolos de cultivos del laboratorio de LeBris S. Quinn Ph.D (Seattle)
5- Descripción de placas multiwell del catálogo de Corning.
6- Hoja informativa del catálogo de ATCC (http://www.atcc.org) sobre C2C12 y L6)

Observación al microscopio

-4X aumentos. Nunca se ve muy nítido porque hay mucha refringencia. Utiliza el filtro del extremo derecho. Está bien para tener una idea de la confluencia y del estado general de la placa.
-10X aumentos. Lo mejor para observar las células: se observa muy bien la morfología y se tiene una idea de la densidad. También se observa bien si hay contaminaciones. Necesita el filtro del extremo izquierdo.
-20X. Para observar con más detalle la morfología. Se ve más oscuro que a 10X. Utiliza el filtro del extremo izquierdo.

MATERIALES

Medio

DMEM high glucose (4,5g/l D-glucose) + L-Glutamina (No HEPES; con piruvato)
*BioWhittaker. Cat# 12-604F 500ml
También puede usarse DMEM sin glutamina y suplementarlo con esta al abrirlo (diluir la glutamina 1:100)
*DMEM no Gln: Biological Industries (distribuido por Reactiva) Cat # 01-055-1A 500 ml. Este es sin piruvato y también hay que suplementarlo
*L-Glutamine solution 200mM: Biological Industries. Cat # 03-020-1B 100 ml
Suplementarlo con la solución de Penicilina-Estreptomicina-Fungizona diluida 1:100.
También puede comprarse el DMEM en polvo. Se diluye se suplementa de bicarbonato, se filtra con un filtro grande acoplado a una bomba y se alicuota en botellas de vidrio autoclavadas de 500 ml, todo dentro de la campana. Conviene tener también polvos como reserva para cuando nos fallan los suministros de DMEM líquido.
Conservación: En nevera o cámara fría, tanto abierto como sin abrir. Tiene fecha de caducidad. Una vez abierta una botella de DMEM no debe usarse más allá de 15 días después o debe volver a suplementarse de glutamina pues esta está prácticamente agotada.
Desechar si la botella está de color muy rosa-fucsia en vez de rojo o si tiene flotadores.
Demasiado rato calentándose al baño de 37°C hace que se descomponga la glutamina y aumente mucho el NH_4^+ del medio.
En los cultivos: si el medio está demasiado rosa fucsia, está basificado y probablemente lleva mucho rato fuera del incubador o hay algún problema con el CO_2 del mismo. Si está amarillo, está acidificado, y puede ser que las células lo hayan gastado y haya que renovarlo o que esté contaminado. Si el medio está turbio probablemente está contaminado de bacterias.
Lote: los DMEM de distintas casas comerciales o de distintos lotes suelen ser bastante constantes porque su composición es fija, pero conviene llevar un seguimiento del lote que estamos usando para localizar alguna alteración o cambio en los cultivos.

Suero

Suero Fetal Bovino (Fetal Obvien Serum, FBS) o Fetal Calf Serum (es lo mismo).
Una vez recibido, descongelar, inactivar por calor, alicuotar en tubos de rosca estériles de 50 ml (Falcon, corning...) y poner cinta de pintor alrededor del tapón.
Conservación : Se conserva a -20°C y aguanta más de una ciclo de congelación y descongelación, pero no conviene hacerlo demasiadas veces. Una vez abierta una alicuota de 50 ml puede guardarse unos dias a 4°C si no se ha usado entera.
Lote: el FBS varía muchísimo y tiene mucha influencia en los cultivos: metabolismo, morfología, viabilidad... ya que contiene los aminoácidos, hormonas, factores de crecimiento, vitaminas, etc... CONVIENE CONTRATAR UN STOCK IMPORTANTE CON LA MISMA CASA

COMERCIAL ¡¡Y EL MISMO LOTE!! de forma que nos dure varios años: se hace una estimación y se reserva de sobras y se van haciendo pedidos y pagando tal como vamos necesitando, no hay que comprar toda la reserva. En caso de que hayamos de cambiar de FBS (lote o casa comercial) se debe pedir primero una alicuota de prueba gratis y probar unas placas en paralelo con nuestros cultivos habituales para ver la diferencia en proliferación, viabilidad, etc...

Horse Serum (HS). Debemos tener las mismas consideraciones que con el FBS, con la salvedad de que es muy poco usado y nadie va a tener para prestarnos. Su color habitual es más claro que el FBS (más amarillo frente al marrón del FBS) y es más pobre en nutrientes, factores de crecimiento, etc...
Nota: si alguna vez nos quedamos sin FBS podemos mantener el stock con HS temporalmente.

Medio de crecimiento: DMEM suplementado con 10% ~~DMEM~~ FBS. Usar para C2C12 y para L6E9, para células de stock y para sembrar células; o sea, para células en proliferación. Sólo cambiamos a otro medio cuando queremos diferenciar las células
Si queremos aguantar un stock con menor proliferación podemos rebajar el % de suero por un período corto de tiempo; pero esta técnica no es muy controlable y a veces las células acaban con mal aspecto, yo prefiero no hacerlo.
Igualmente, podemos aumentara 20% FBS cuando necesitemos estimular la proliferación (por ejemplo para células recién descongeladas o para muy muy diluidas).
Abrir la botella de 500 ml dentro de la campana, añadir 5 ml del antibiótico (PSF) 100X, 5 ml de glutamina 100X (si el medio no la lleva ya) y 50 ml de FBS (si no necesitamos tanto, añadir el FBS correspondiente aparte: falcon, botella de 100ml... Remover cogiendo del cuello de la botella y describiendo círculos con el culo, no volcar bocarriba y bocabajo por que el líquido que quede en la rosca del tapón es una posible vía de contaminación.

Siempre sacar el medio con pipeta y nunca introducir una pipeta que se ha usado en otra botella.

PBS:

Para 1 litro:	NaCl	8g
	KCl	0,2g
	KH_2PO_4	0,2g
	Na_2HPO_4 ($2H_2O$)	1,43g
	ó	
	Na_2HPO_4	1,14g

Ajustar a pH 7,4 +/- 0,05 con HCl y autoclavar

Preparar al menos 2 litros, repartir en botellas de vidrio de 500ml y autoclavar. También se puede preparar 10X e ir diluyendo, pero así a veces precipita, en ese caso calentar a 37°C para disolverlo. Conservar a 4°C o a temperatura ambiente
NOTA: Para tripsinizar mejor puede suplementarse con Versene (EDTA comercial)

*VERSENE 100ml EDTA solution Cat# 17-711A BioWhittaker
NOTA: Las células pueden aguantar bien en PBS varios minutos.

Tripsina: Utilizar Tripsina-Versene (EDTA)
*TRYPSIN VERSENE 200 mg/L versene (EDTA)-500 mg/L trypsin 1:250
BioWhittaker. Cat #17-161E 100 ml
El EDTA quela los cationes divalentes inhibiendo la adhesión celular a la placa e intercelular. La tripsina sin EDTA funciona peor. Una vez recibido alicuotar en tubos estériles (Falcon) de 15 ml, rodear el tapón con cinta de pintor y guardar a -20°C. Puede congelarse y descongelarse varias veces, aunque puede perder algo de actividad. Si se queda uno o dos días a 4°C todavía funciona. A veces la tripsina no funciona o lo hace muy lentamente sin causa aparente: entonces, añadir más volumen o, si es posible, usar otra alícuota.
Pancreatina: Puede usarse igual que la tripsina tanto con C2C12 como con L6E9. Es mucho más rápida.

Viales de congelación. Los hay de 1, 2 o 3 ml. NUNC o Corning son buenos. Los NUNC tienen la ventaja de que se sostienen de pie porque la base es plana y no redondeada como la de los otros. Yo utilizo NUNC 1ml para las líneas celulares normales y viales de 2 ml para Yoshida AH-130
*NUNC Labclinics Cat# 366656 1ml

Placas y multiwells. Para C2C12 y L6E9 se usan placas de plástico normal y no hace falta que tengan ningún recubrimiento especial. Costar funciona bien, aunque Falcon (en especial he probado las multiwell 6(MW6)) va mejor para C2C12.
Ver hojas adjuntas al final para medidas de superficie y volúmenes.
-Placas 15 cm θ. Para producción a gran escala; ninguno de los ensayos que yo he realizado
-Flascones de 75cm^2. Para mantener stock. Seguros frente a contaminación pero caros. 10 ml de medio
-Placas 10 cm θ. 75cm^2. Para stock y para RNA. Precaución de que el medio no manche la tapa. 7-10 ml de medio.
-Placas 6 cm θ. Para recoger proteína en gran cantidad, para RNA con tripure (o casi con el método normal si no hace falta mucho). 4 ml de medio.
-Placas 3,5 cm θ. Para recoger proteína, para inmunocitoquímica, para síntesis y degradación... El tamaño es igual a un pocillo de una MW6, la ventaja es que se puede individualizar cada muestra, pero los inconvenientes son suficientes para pasarse a MW6: hace falta una bandejita esteril para poner en el incubador, es muy facil que se salga el medio y manche la tapa y los bordes (y por ello que se contaminen) son dificiles de manejar y es facil que se caigan. 1,5-2 ml de medio.
-MW6. Fáciles de manejar, cómodas y seguras frente a contaminaciones. Para recoger proteína, para inmunocitoquímica, para síntesis y degradación... 1'5-2 ml de medio.
-MW12. 1-1,5 ml de medio. Para incorporación de timidina...
-MW24. Cómodas de manejar pero muy mala visualización al microscopio. Para cuantificar proteína total y otros ensayos que no requieran mucha muestra ni manipulación. 0,5-1ml de medio
-Con otras MW más pequeñas no podría asegurar que las monocapas crezcan bien
En el carrito guardar las placas dentro de su bolsa lo más herméticamente posible cerradas con cinta.

Se deben tripsinizar cuando están a 60-70% de confluencia. EL STOCK NUNCA DEBE LLEGAR A CONFLUENCIA. Si estando a menor confluencia se observan zonas de clones también se debe tripsinizar, para que no empiecen a diferenciarse las del centro.

Sembrar: -$1x10^5$ cels/pozo de multiwell 6 o placa de 3,5 cm Ø.

-$0,2x10^5$ cels/pozo de multiwell 24

-$0,7x10^6$ cels/placa de 10 cm Ø

Dejar incubar 48h y cambiar a medio de diferenciación. Comprobar que la confluencia es del 80%.

Se diferencian en 2% FBS. Los miotubos están establecidos en 4 o 5 días y aguantan de 7 a 9 días.

Peculiaridades:

Después de tripsinizar conservan aspecto refringente y poco estrellado durante un día al menos, y les cuesta arrancar a proliferar.

NO COMPARTIR PIPETAS NI MEDIO ENTRE LAS LINEAS CELULARES Y PROCURAR NO TRABAJAR CON LAS DOS A LA VEZ Y, MEJOR AUN, NO SIMULTANEARLAS EN LA CAMPANA, PARA EVITAR CONTAMINACION CRUZADA: que las células de un tipo crezcan en los cultivos de las otras

TECNICAS

Stock. Mantenimiento

Son las placas que se mantienen siempre en proliferación como fuente para sembrar experimentos. Siempre va en DMEM + 10% FBS.

Debemos mantener siempre para stock varias placas en paralelo a las que tengamos para experimento. Si no tenemos pensado realizar experimentos en varias semanas es mejor no tener stock en marcha ya que después de descongelar se puede volver a tener stock suficiente para sembrar en una semana más o menos.

El stock se puede mantener en placas de 10cm Ø o en frascos (flascones) de 75 cm^2. Las primeras se pueden manejar mejor para tripsinizar y son más baratas y los segundos son más seguros respecto a evitar contaminaciones.

Es conveniente no mantener el stock más allá de los 15-17 pases (tripsinizaciones), al menos para C2C12 y L6E9. Es fácil que después de tantos pases las células cambien algunas de sus características y empiecen a transformarse.

No se debe dividir a diluciones mayores de 15-20 X ya que las células quedarán muy aisladas en la placa y empezaran a crecer por clones que quedaran muy apretados de células (con lo que pueden empezar a diferenciarse las del centro) cuando aún queda mucha placa vacía. Además cuando las células están muy diluidas tienen dificultad para arrancar a proliferar. Entre 5 y 15 es una buena dilución para mantener el stock. Se puede hacer un cálculo considerando que más o menos se duplican cada día, pero esto no es muy exacto.

Conviene mantener el stock dividiendo unas 2 o 3 veces por semana, lo que significa diluciones entre 5 y 15 X.

Aproximadamente, una placa de 10cm Ø con 200.000 células C2C12 tarda unos 4 días en estar lo bastante llena para tripsinizarse otra vez, pero lo que hay que hacer es, cada temporada/línea/experimentador calcular los días necesarios para llegar a la confluencia sembrando a diferentes densidades.

Para mantener el stock de rutina basta tener unas tres a cinco placas (una sola corremos el riesgo de que le pase algo) y si hay que sembrar necesitaremos más.

Para sembrar un experimento debemos hacer un cálculo de las células que necesitamos y sembrar unos días antes las placas necesarias para que cuando estén aproximadamente a 80%-90% de confluencia (C2C12 = 3,5-4 x 10^6 cels) tengamos suficientes para sembrar.
Es bastante útil tener varias placas a diferentes densidades cuando no sabemos exactamente cuando las vamos a necesitar, si necesitaremos más o menos células o que dia podemos dividir.

Cambiar el medio

A las placas de stock, que no suelen estar muy llenas, se les puede cambiar cada 2 a 4 días. A las placas para experimentos se les cambia idealmente cada 2 días (o sea, un día sí y otro no). Normalmente se puede dejar un día más durante el fin de semana, no pasa nada.
El medio se retira con una pipeta pasteur estéril, inclinando la placa y aspirando del lateral sin tocar las células. Hay que procurar no quitar del todo la tapa de la placa y orientarla hacía la llama del mechero.
Para reponer el medio pipeteamos de la botella un ml más de lo necesario que no se suelta en la placa sino que se devuelve a la botella (las burbujas de líquido que a veces se forman al soltar todo el contenido de la pipeta ponen en contacto la placa con la pipeta y pueden ser vía de contaminación).
No utilizar la misma pipeta para botellas diferentes.
Pipetear siempre y no verter directamente de botellas o tubos.

1. Medium vol.

Dividir (Tripsinizar)

2. Tryp

3. Cell seed.

Tryp 2ml

Opción 1

-Lavar las placas 2 o 3 veces con PBS (tres veces mucho mejor, ya que los restos de suero inhiben la tripsina).
-Añadir de 0,5 a 1 ml de tripsina a la placa. p150 → 3mL T75 → 2-3mL
-Meter en el incubador de 3 a 7 minutos.
-Comprobar en el microscopio que las células se han despegado (se ven como esferas muy refringentes que nadan por el medio).
-Se pueden dar unos golpecitos secos al costado de la placa para ayudar a que se desenganchen.
-Recoger las células en 5-10ml de medio con 10% de suero y pasarlas a un falcon. Conviene hacerlo en varias veces para lavar la superficie de la placa porque si no se pueden quedar muchas allí.

3X. 6mL medium → FBS

-Centrifugar 4-5' a 1000-1500 rpms (220g). 1500 rpm 5 min 20°C. 1200 rpm
-Aspirar el medio y poner medio fresco.
-Resuspender con la pipeta normal cogiendo y soltando el medio contra las paredes o apretando contra el fondo unas 20-30 veces; o con una pipeta pasteur larga con una perilla en el extremo, realizar la misma operación 100 veces al menos: coger y soltar contra las paredes. Esta última manera es con la que se consigue mejor resuspensión. Es muy importante que no queden grumos.

Opción 2

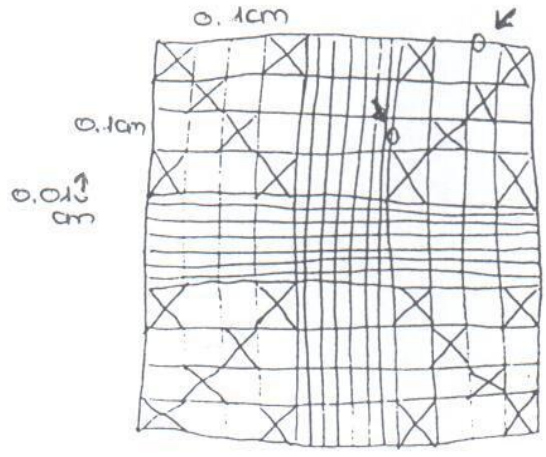
-Igual que la anterior hasta que añadimos 1ml de tripsina
-Metemos en el incubador 1 minuto exactamente.
-Sacamos y aspiramos la tripsina sin apurar del todo
-Dejamos varios minutos en el incubador, hasta que se han desenganchado.
-Recogemos las células en medio con suero, también en varias veces, pero ahora no hace falta centrifugar, ya que hemos quitado la tripsina.

Sembrar

-Tripsinizar y recoger las células de varias placas (2 a 8) juntas en un falcon, en un volumen conocido: 10-15 ml.
-Resuspender muy muy bien pipeteando 100-150 veces con una pipeta pasteur larga con perilla
-Coger una gota (unos 15 µl) y ponerla en la cámara de Neubauer.
-Contar en el microscopio y calcular las células/ml que tenemos. Si se observan grumos volver a resuspender y contar.
-Coger el volumen necesário para sembrar las placas que queramos y diluirlo hasta el volumen final que pondremos en las placas en otro tubo. Cerrar y remover por inversión varias veces.
-Todas las placas de un experimento conviene que estén sembradas de la misma dilución, ya que nunca prepararemos dos exactamente iguales. Si no nos cabe todo el volumen que necesitamos, preparamos las células al doble de concentradas y ponemos al pocillo la mitad de volumen del que corresponderia y la otra mitad se rellena con medio fresco.
-Durante la siembra tapar y agitar por inversión el tubo con las células cada una o dos veces que saquemos con la pipeta.
-Observar muy bien el volumen que dejamos salir de la pipeta y utilizar la pipeta que más se ajuste al volumen que estamos sembrando.
-Una vez sembradas las placas, agitarlas arrastrando horizontalmente (arrastrándolas en la superficie de la campana) hacia delante y hacia atrás, y hacia izquierda y derecha; también volcándolas un poquito en estas dos direcciones, pero nunca describiendo círculos ya que así se concentran en el centro.

Contar células

Poner 15 µl entre la entre la cámara y el cubre. Contar las células que hay en los 6 cuadritos señalados en el dibujo de cada uno de los cuadrantes. De las células que tocan las líneas exteriores del cuadrante se cuentan solo las que lo hacen en la línea de arriba y en la de la derecha.



15-25/30

El número de células en los 6 cuadritos multiplicado por 4 da el número de células en un cuadrante.
La media de los cuatro cuadrantes x 10^4 = nº cels / ml.
El cubre que debe usarse es uno especial para esto, rectangular y grueso que hace que las células no se muevan.

Centrifugar

Para separar las células del medio sin dañarlas, centrifugarlas 3-5 minutos a 220g (en la centrífuga de la sala de cultivos 1000-1500 rpms) a temperatura ambiente.

Congelar

-Tripsinizar, contar y resuspender en medio de congelación a 10^6 cels/ml
-Repartir en viales de congelación a 1 ml/vial
-Dejar 1h a 4°C
2h a –20°C
o/n a –80°C
-Conservar en N^2 líquido

Medios de congelación

1. DMEM – 20%FBS + 10%DMSO
2. DMEM –10%FBS + 5%DMSO (Recomendado por ATCC para C2C12)
3. FBS-10%DMSO (Recomendado por Perla Kaliman para L6E9)

En mi experiencia, todos estos van bien.
La congelación secuencial descrita arriba es la ideal, pero también puede hacerse directamente a –80°C o Nitrógeno.

Conservación en congelación

Para asegurarse de la integridad de la línea conservar siempre en N_2 líquido. Congelar los pases más tempranos posibles y a ser posible conservar alícuotas en tanques diferentes, para evita accidente (por ejemplo en del grupo Cancer en el de la unidad A del departamento, en la Facultad de Químicas).
Procurar no mantener a –80°C más de una semana o dos. Más tiempo no implica que las células se hayan estropeado, pero existe la posibilidad y no es fiable.

Descongelar

-Tomar el vial del tanque de nitrógeno y descongelar rápidamente en un baño a 37°C agitando con la mano. Procurar que no se moje el tapón.
-Resuspender en medio con suero.
-Centrifugar, aspirar el medio y resuspender en medio fresco.
-Sembrar en una placa de 10 cmθ.
Las células recién descongeladas suelen tardar un poco más en arrancar a proliferar. Las C2C12 y las L6E9 que tenemos en el grupo tienen una viabilidad muy alta y enseguida llenan la placa y crecen bien.

Inactivación del complemento

Es una medida preventiva que se toma con el suero para que el complemento no reaccione y dañe a las células. Consiste en, una vez descongelado el suero, antes de alicuotar, calentarlo 20-30' a 55°C en un baño.

Pipetas Pasteur. Siempre autoclavadas en botes de vidrio o bolsas de autoclave. Abrir cerca de la llama y cogerlas por el extremo opuesto a la punta. Agitar un poco con un movimiento seco para que salgan los extremos de las Pasteurs. Tocar sólo la pipeta que se saca, si se ha tocado alguna otra, sacarla también. Cuidar mucho de que no se ha rozado nada con la punta y pasear rápidamente por la llama como precaución de todas formas. Se usan para aspirar el medio y para resuspender células. En este último caso las largas van mucho mejor.

Rasquetas. Son como palas pequeñitas de plástico que se compran estériles y se usan para recoger bien todas las células o todo el lisado de una placa. Las hay de varios tamaños y las que van mejor son unas totalmente planas de una sola pieza. Si no hace falta que sean estériles se pueden reutilizar.

Antibióticos

-PSF. Peniclina Estreptomicina Fungizona. Los dos primeros son anti-bacterias y el tercero anti-fúngico. Se añaden siempre al DMEM justo al abrir la botella. Se compra ya mezclado 100x. Se conserva alicuotado en 10 ml a –20°C. Resiste varios ciclos de congelación-descongelación

*BioWhittaker:Penicillin(10000U/ml)-Sterptomycin(10000µg/ml)-Fungizone(25µg/ml)Mixture 100 ml. Cat# 17-745H

-Gentamicina. Para eliminar contaminaciones de Bacterias (ver contaminaciones). Se conserva 4°C. No debe usarse rutinariamente ya que se acaban por crear Bacterias resistentes.

*Sigma 50mg/ml 10 ml Cat# G1397

Contenido del carrito

-Pitetas de 2, 5, 10 y 25 ml
-Placas de 10 cm Ø y flascones grandes
-Multiwells de 6 y 24 pocillos
-Pasteurs autoclavadas
-Tubos de rosca estériles de 50 y 15 ml
-Eppendorfs autoclavados (en bote cristal)
-Cajas de puntas amarillas y azules autoclavadas
-Cinta de pintor
-Filtros de 22µms
-Cámara de Neubauer y cubres
-Jeringas de 5, 10 y 50 ml
-DMSO estéril
-Viales de congelación
-Tripán blue
-Soportes de Porexpan de los falcons de 50 y 15 ml

Contenido del frigorífico

-DMEM
-FBS, HS (si ya están empezados)
-PBS
-Gentamicina

CONTAMINACION

Bacterias

Es la contaminación más frecuente y el contagio es fácil incluso solo por compartir incubador con otras placas contaminadas. Se observa al microscopio como unos puntitos o palitos, o grupitos de estos, que están flotando en el medio o adheridos a la célula. Los que flotan se enfocan un poco más arriba con el microscopio que las células. A simple vista se distingue porque le medio se pone amarillo y/o turbio. Cuando detectemos contaminación de bacterias lo mejor es desechar las placas contaminadas y los medios que se han utilizado con ellas y descongelar stock nuevo. Si

queremos conservar las placas debemos usar Gentamicina, como antibiótico no habitual, como tratamiento (no añadir rutinariamente Gentamicina a los cultivos porque crearemos Bacterias resistentes). Se debe:

-Lavar 2-3X las placas con PBS suplementado con Gentamicina 100µg/ml

-Poner en medio con Gentamicina 100µg/ml 24 a 48h.

-Para los días siguientes lavar con PBS+Genta entre los cambio de medio y mantener en 50µg/ml de Gentamicina.(el antibiótico a tan alta concentración (100) puede dañar las células)

Si hemos detectado la contaminación cuando ya es muy fuerte desechar las placas directamente: las células ya están dañadas.

Hongos

Se observan como plaquitas negras de diversos tamaños, bastante más grandes que las células, que van aumentando de tamaño día a día. Se pueden intentar eliminar aumentando al doble la concentración de lo habitual de fungizona o mecánicamente lavando varias veces las placas con PBS a chorro fuerte desde la pipeta.

Levaduras

Que yo sepa no se pueden eliminar. aparecen como bolitas o palitos refringentes que se diferencian de los mioblastos en división porque son de tamaño inferior y porque presentan gemación (este es el mejor rasgo para reconocerlas).

Micoplasma

Los micoplasmas no se ven a simple vista ni al microscopio. solo cuando la contaminación es muy avanzada se distinguen puntos negros en el citosol de la célula. Aunque se venden productos para eliminarlos es muy dificil deshacerse de esta contaminación que, además es muy peligrosa: se instala en las lineas celulares, se transmite con los pases y cambia sus caracteristicas: normalmente disminuye la proliferación, la viabilidad, se hace casi imposible llegar a confluencia... pero además afecta al metabolismo normal de cada línea celular de forma que puede ser diferente, y empezaremos a tener comportamientos y resultados raros.

Para comprobar si tenemos micoplasmas podemos hacer una tinción especifica de DNA (por ejemplo con BrEt y mirar en un microscopio de UV de más de 80 aumentos, pero la única prueba concluyente es una PCR).

Kit detección de micoplasma: *Biological Industries Co. Reactiva S.A. Cat# 6601-A

Se debe hacer una comprobación rutinaria de micoplasma de las líneas celulares que tengamos (una vez al año o algo así)

NORMAS ESTERILIDAD

-Procurar no meter cosas muy sucias en la sala de cultivos: cajas y paquetes, la bata muy sucia, material para otros experimentos... solo lo que necesitemos para cultivos. Sobre todo cosas que hayan estado en contacto con animales o bacterias etc...

-Mantener siempre cerrada la puerta de la sala.

-Nada más entrar lavarse las manos con jabón y mojarselas bien con etanol (es un etanol diluido que ya está preparado en cultivos en la garrafa grande). Cada cierto rato o cuando tocamos algo más sucio como el agua del baño o el frigorífico volvemos a ponernos más alcohol.

-Tener el material de cultivos preferentemente en la sala, en el carrito o en le frigorífico, porque allí pasan las noches al U.V.

-Al encender la campana fregar bien con etanol la superficie de trabajo, aunque acabe de hacerlo el que ha trabajado antes. Si llevamos mucho rato trabajando pasamos un poquito de alcohol por donde hemos estado trabajando.

-Pasarle alcohol a la superficie de las botellas, al pipetus y a otros objetos que utilicemos antes de meter en al campana (más que nada si vienen del baño, del frigorífico o de fuera de cultivos.

-Al abrir el incubador procurar no hablar mirando hacia dentro del mismo y no mantener mucho rato la puerta abierta.

-Las pipetas que se han abierto quitando el plástico de la parte de la punta en vez de la parte superior, o si han tocado o han podido tocar cualquier cosa con la punta, se desechan.
-Organizarse la disposición de forma que las botellas de medio y las células queden cerca de la llama, y las cosas cerradas que no estamos usando más lejos.
-NUNCA PASAR LA MANO POR ENCIMA DE BOTELLAS O PLACAS ABIERTAS
-No compartir pipetas entre botellas.
-Al abrir un falcon o una botella poner el tapón boca abajo (suponemos que la superficie está estéril, la hemos lavado con alcohol) y al ir a cerrar pasar cerca de la llama el cuello de la botella o del tubo y el tapón. Por últimos para guardarlos rodear el filo del tapón con cinta de pintor.
-Guardar las bolsas de placas, tubos etc.. lo mejor cerraditas y herméticas posible, cerrando con cinta de pintor.
-Pasar las pipetas Pasteur por encima de llama (muy muy ligeramente) antes de usar.
-No abrir las placas fuera del incubador.(Pero el hecho de que se abran por cualquier cosa no significa que se han contaminado ni mucho menos)
-Retirar con la pasteur y la bomba el liquido que haya en las tapas o en los laterales de fuera de las placas.
-La poyata donde está el microscopio la suele limpiar la mujer de la limpieza pero si la vemos sucia, la limpiaremos con un poco de lejía diluida y etanol.
-Procurar tener cuantas menos cosas a la vez mejor dentro de la campana
-ANTE LA DUDA SOBRE LA ESTERILIDAD DE CUALQUIER COSA, DESECHARLA

EL INCUBADOR

Elementos a controlar:
-Humedad. Mirar que el monitor indica la humedad de referencia (90%). Hay que comprobar de vez en cuando que la botella con agua que hay en la parte trasera del incubador tiene al menos tres o cuatro dedos de agua. Esta agua es estéril y está en una botella que indica "cultivos".
-Temperatura. Debe estar a 37ºC.
-CO_2. Si baja de la concentración de referencia (aprox 4,5%) comprobar las botellas que están en el almacén; deben estar a 1,75 Bars

Limpieza
Debe limpiarse cada mes o dos meses, o cada vez que haya contaminación generalizada o huela mal. Hay que desmontar todas las bandejas y piezas de soporte, lavar con estropajo, agua y jabón, enjuagar con agua, fregar con etanol y finalmente, rociar con el spray mata-lo-todo (botella blanca y verde).

CAMPANA

En al parte superior de la campana hay cuatro botones; de izquierda a derecha: ON/OFF de la ventilación, "stand-by/funcionando" de la ventilación, la luz y, por último los Ultra-Violeta. El botón de ON/OFF (el primero de la izquierda) no se toca, se deja siempre en ON.
Al empezar a trabajar poner en marcha la ventilación (las lucecitas que indican el nivel de ventilación deben llegar al 70-80% de altura, hay una rayita indicativa), encender la luz y apagar los UV (¡Ojo! Comprobar que están apagados porque con la luz encendida no se nota si los UV están encendidos simultáneamente y mucho rato trabajando así nos puede dañar la retina). Fregar la superficie con etanol generosamente y repartirlo con un papel y encender el mechero (abrir la palanca del tubo, que queda perpendicular cuando está abierta, y la rosca). Nunca abrir el mechero cuando la ventilación está cerrada o quemaremos el filtro.

La campana debería limpiarse con detenimiento una vez al mes para ir bien. Consiste en desmontar la bandeja y limpiarlo todo con 1: jabón y estropajo, 2: etanol, 3: líquido desinfectante extra-fuerte (botella blanca y verde que hay en cultivos).
Para desinfectar después de trabajar dejar los UV 15' al menos. Menos rato no es efectivo desinfectando, así que mejor nada, y mucho rato tampoco es recomendable ya que la lampara UV tiene vida limitada y es muy cara.

LA BOMBA

Para que aspire bien hay que comprobar que el tapón del quitasato esta bien (pero bien) metido al igual que el tapón de la trampa (el quitasato o la botella que hay conectada después) y que el tubo no está doblado o pisado por algo. La bomba es una buenísima fuente de contaminación, no tocarla mucho.
Al acabar de trabajar aspirar lejía directamente con el tubo de la botella de lejía diluida que hay en cultivos y luego aspirar un poco de agua (para que la lejía no se coma el plástico del tubo). El contenido del quitasato debe quedar violeta o blanco. Volcarlo en el fregadero (enjuagar luego el fregadero) y volver a colocar el quitasato en su sitio.
Si los tapones están bien metidos y el tubo no está doblado pero la bomba no tira debe ser cosa del motor.
El interruptor de la bomba esta en la parte de fuera de la campana justo encima de una de las patas.

PEDIDOS

Hacer los pedidos con bastante antelación porque la puntualidad de las distribuidoras no es muy fiable. Especificar bien el número de catalogo de lo que pedimos (y de lote en caso de que tengamos uno contratado) y si tenemos prisa especificar URGENTE en letras gigantes en la hoja de pedido y mejor, llamar también a la casa comercial para apretarles. No hace falta hacer demasiado acopio de cosas, así no se nos queden caducadas u olvidadas por ahí. Cuando lleguen las cosas colocarles las letras CANCER bien visibles en la etiqueta o en la caja (aún así su desaparición no está garantizada).
Llevar un registro de los pedidos hechos (fotocopia de las hojas de pedido o apuntado en un papel) y de lo recibido (albaranes o apuntado en un papel) para reclamaciones y control interno nuestro. De todas formas Las secretarias archivan las hojas de pedidos y los albaranes de todos lo que se pide.
Para cualquier duda preguntar a Raquel (secretaria)

MATERIAL LABORATORIO

Traer del laboratorio, mejor en una bandeja: pipetus (el succionados automático para pipetear), rotuladores, cámara de Neubauer, micropipetas calculadora, libreta laboratorio, bolígrafo, cronómetro, ...
Si queremos usar algo del laboratorio regularmente en cultivos, como una bandeja para meter en el incubador, debemos lavar con jabón, fregar con etanol y poner dentro de la campana bajo los UV durante un rato.

NORMES DE TREBALL A LES SALES DEL SERVEI DE CULTIUS CEL·LULARS.

Els usuaris, per incorporar-se al servei, hauran de llegir i signar la conformitat amb la normativa d'utilització.
Aquesta normativa serà respectada pels usuaris en totes les ocasions.

1. **Registre d'utilització del servei.**

Els usuaris hauran d'anotar en el llibre d'inscripcions del servei el temps de reserva estimat d'us de les cabines.
Els usuaris hauran de anular amb antelació, sempre que sigui possible, les reserves que no es faran efectives.
Els usuaris hauran d'anotar, en el full de treball de les cabines, el temps real d'utilització.

2. **Vestimenta.**

Es imprescindible utilitzar bata, proporcionada pel servei, per fer manipulacions a les instal·lacions. La bata es col·loca abans d'iniciar les manipulacions i es retira i deixa en el servei en acabar.

3. **Instruccions de treball en les cabines.**

Abans de començar i, en acabar, renteu la zona de treball amb alcohol del 70%.
Abans d'iniciar el treball, poseu en posició de màxima potencia la ventilació de la cabina i enceneu el llum.
Abans d'iniciar el treball comproveu el nivell del líquid en el kitasato de manera que no superi el nivell marcat
No deixeu les pipetes en posició invertida quan continguin líquid.
Deixeu els pipetejadors penjats en els seus corresponents suports i endolleu el carregador quan s'acabi la manipulació.
No intercanvieu el material auxiliar (pipetes, gradetes, etc.) entre cabines a sales del servei.
No llenceu material amb líquid a les escombraries destinades a ser autoclavades, ni material fora de la bossa.
Canvieu les bosses per autoclavar si són plenes; deseu-les al contenidor de sala d'autoclau per a la seva esterilització.
Dipositeu les pipetes Pasteu de vidre usades en el contenidor de plàstic groc identificat per a aquest fi (agiteu el contenidor per optimitzar la cabuda de pipetes).
Dipositeu les agulles de xeringues en el contenidor groc identificat per aquest fi.
En acabar el treball a la cabina i, per tal de desinfectar el circuit de tubs, aspireu lleixiu al 20%.
Buideu el kitasato si supera el nivell marcat.
En acabar el treball a la cabina, col·loqueu la ventilació en posició de reducció.
Tanqueu el sistema de buit un cop hagueu acabat de treballar en la cabina.

4. **Notificació d'incidències.**

Notifiqueu per escrit, en un full d'incidències, el mal funcionament de qualsevol aparell del servei.
Notifiqueu per escrit, en un full d'incidències, la detecció de contaminacions.

5. **Treball als incubadors.**

Abans de començar la manipulació al incubador, renteu-vos les mans o els guants amb alcohol del 70%.
Retoleu tot el material de plàstic que deixeu en els incubadors amb el nom de grup i la data.
Notifiqueu a la tècnic si hi ha algun material oblidat, vessaments o altres fons de contaminació
No intercanvieu el material entre incubadors o sales del servei sense prèvia autorització.

6. **Desinfecció i cura de les sales.**

En entrar a la sala, si està oberta la llum UV tanqueu-la immediatament.
Netegeu l'àrea de treball al voltant del microscopi amb alcohol del 70% abans i després d'haver-hi treballat.
L'últim usuari del dia haurà de connectar els llums UV de les cabines i de les sales de cultius.
L'últim usuari del dia haurà de tancar els banys i comprovar que el microscopi esta apagat.
No emmagatzemeu ampolles en les neveres, manteniu només aquelles que heu d'utilitzar immediatament.

HE ENTÈS LES NORMES I ESTIC D'ACORD

SIGNAT (Nom, cognoms, grup de recerca i Departament)

(Fac. Biología, Servei de cultius Cel·lulars

NORMAS DE TRABAJO Y MANTENIMIENTO EN LAS SALAS DEL SERVICIO DE CULTIVOS

Cabinas de flujo laminar y sala

1. Para el buen funcionamiento de las cabinas no debe dejarse ningún tipo de material dentro de ellas (placas,botellas, tubos, flascones, puntas de pipetas, etc).

2. El material fijo de la cabina como gradillas y pipeteadores no deben ser sacados fuera de las cabinas ni untercambiarse entre las diferentes salas del Servicio.

3. Antes y después de trabajar debe pulverizarse la zona de trabajo, primero con agua y posteriormente con alcohol al 70%.

4. El pipeteador no debe colocarse invertido con pipetas que contengan o hayan contenido líquido, dado que estropea el filtro.

5. El filtro del pipeteador se cambia cuando se moja, al igual que la bateria.

6. Es peligroso dejar las llaves del gas sin cerrar.

7. Antes y después de trabajar en las cabinas, **debe comprobarse el nivel de líquido del contenedor**. Si supera el nivel marcado debe vaciarse. Una vez vaciado, deben vertirse unos ml de lejia los cuales se dejaran como desinfectante de los líquidos aspirados. En caso de que el filtro resulte mojado, debe cambiarlse por otro (están en el segundo cajón). El filtro mojado no debe tirarse, dejadlo en la pica.

8. Al final del trabajo en la campana debe aspirarse siempre lejía al 20% y agua a continuación, para desinfectar el circuito de tubos.

9. No puede tirarse en la basura, que se esteriliza en el autoclave, ningún material con líquido, y cuando esté llena, la bolsa debe cambiarse.

10. En los contenedores de vidrio sólo se pueden tirar las pipetas Pasteur aseguraos que la entrada no quede obturada por las pipetas, agitando el contenedor.

11. El área de trabajo alrededor del microscopio debe mantenerse limpia con alcohol 70%.

12. Las botellas de alcohol 70% o lejía 20% se deben rellenar cuando se terminan.

13. Las botellas vacías de medio se deben lavar varias veces con agua destilada y colgarlas en el escurridor.

14. Para la preparación de medios y PBS existe una lista, en la cual todos los usuarios deben apuntarse con la suficiente previsión y anticipación.

15. Existe un libro para la reservar cabina. Reservad sólo el tiempo estimado de uso y sed puntuales.

16. Apuntad el tiempo real de trabajo en las hojas de las cabinas.

17. Es obligatorio el uso de batas durante el tiempo de trabajo en la sala.

18. El último usuario del día debe encender las lámparas ultravioleta de todas las cabinas y salas antes de abandonar el servicio.

Incubadores:

1. Antes de abrir el incubador pulverizaos las manos con alcohol 70%.

2. Hay que controlar todas las placas que se tienen en el incubador. Si hay placas olvidadas dar aviso.

3. Todo material debe ser identificable.

4. En caso de contaminación debe darse aviso a la persona responsable del servicio.

Incubadors

1. -Polvoritzeu les mans **amb alcohol al 70%** abans d'obrir els incubadors
2. -Marqueu amb la data, el nom de l'investigador i el del grup de recerca tot el material que dipositeu als incubadors. El material no marcat serà retirat.
3. -Controleu el material dipositat amb regularitat.
4. -Aviseu a la tècnic si sospiteu que hi ha material oblidat, vessaments, o altres fonts de contaminació.
5. -Aviseu immediatament a la Tècnic del Servei i al responsable de cultius del grup de Recerca en cas de contaminació del material particular o general dipositat als incubadors. Apunteu el tipus de contaminació. LLENCEU EL CULTIU INMEDIATAMENT.

Autoclau

1.- Tot el material de cultius s'ha d'autoclavar a l'autoclau del Servei de Cultius

2.- Per esterilitzar el material, s'ha d'utilitzar un protocol en dues etapes (2 x 10 min a 130ºC). L'objectiu és eliminar durant la segona etapa les possibles contaminacions resultants de l'eventual desenvolupament de les espores que es pugui produir durant la primera etapa.

CALENDARI DE LES TASQUES A REALITZAR RUTINÀRIAMENT AL SERVEI DE CULTIUS

Son tasques que realitza la Tècnic del Servei.
En cas excepcional d'absència de la Tècnic per causes justificades, seran realitzades pels usuaris del grups anotats entre parèntesi.

-Neteja de campanes: 1 cop / mes (1ª setmana) **Un usuari de cada grup de recerca col.laborarà de manera rotatòria en la neteja de la campana que utilitza normalment. (Cada grup la campana que utilitza)**

-Repassar Campanes. 1 cop/setmana (dilluns) **(Cada grup la campana que utilitza)**

-Neteja incubadors sala general (desmontant interior): 1 cop al mes(2ª setmana) **(Bioq. Integrativa i Sucres)**
-Repassar i revisar nivell d'aigua (aigua milliQ autoclavada) 1 cop/setmana (divendres) **(Adenos)**
-Revisar nivell CO2 1 cop /setmana (divendres) **(Pàncreas)**

-Neteja incubadors sala virus (desmontant interior i dipòsit de l'aigua) : 1 cop al mes (3ª setmana) **(Pàncreas)**
-Repasar i revisar nivell d'aigua (aigua milliQ autoclavada) 1 cop/setmana (divendres) **(Pàncreas)**
-Revisar nivell CO2 1 cop /setmana (divendres) **(Pàncreas)**

-Recollir brossa de plàstic per autoclavar i **revisar kitasatos** (si són plens: avisar al responsable de l'últim grup que ha ocupat la campana: cada dia en arribar i avans de marxar (si són plenes) i cada cop que sigui necessari. **(Biomembranes)**

-Retirar contenidors pipetes Pasteur de vidre (verifiqueu que siguin plens): cada dia en arribar i al marxar i sempre que sigui necessari. Deixar a la terrasa. Trucar al responsable de seguretat (Alex, tel: 21109) **(Receptors)**

-Revisar bombes i pipetboy: 1cop/setmana (dimecres) **(Receptors)**

-Neteja i manteniment de microscopis i càmera fotogràfica: 1 cop al mes (4ª setmana) **(Adenos)**
-Netejar i mantenir nivell dels banys d'aigua: 1cop/setmana (dilluns) **(Adenos)**
-Autoclavar bosses de brossa de plàstic: cada dia **(Sr. Joan i Sr. Manolo)**
-Omplir contenidors d'aigua MilliQ, lleixiu 20% i alcohol: cada dia **(Sr. Joan i Sr. Manolo)**
-Revisar centrífugues, neveres i congeladors: cada setmana (dijous) **(Sucres)**
-Retirar material no identificable (material i usuari) de neveres, incubadors i campanes: cada setmana (dijous) **(Sucres)**

-Realitzar compres: alcohol, cel.lulosa, bosses autoclau, CO2, **(Receptors)**
-Coordinació de la utilització del Servei **(Responsable Científic)**
-Sol.licitar revisió sala general cada tres mesos
-Sol.licitar revisió incubadors: 1 cop/ any (mes de març) **(Responsable Científic)**
-Sol.licitar revisió campanes: 1 cop/ any (mes de març) **(Responsable Científic)**
-Manteniment de la llista de telèfons d'urgència o d'avaria d'instal.lacions i d'aparells **(Cada grup de l'aparell que li toqui i Responsable Científic)**
-Indicacions clares i precises de l'actuació en cas d'emergència: CO2, humitat, aire condicionat **(Cada grup de l'aparell que li toqui i Responsable Científic)**

ÁREA DE CRECIMIENTO EN PLACAS

Placas multipozo	Área (cm^2)
6 pozos	9,03
12 pozos	3,66
24 pozos	1,91
96 pozos	0,31
Placas individuales	**Área (cm^2)**
40 mm Ø	9,2
60 mm Ø	22,1
100 mm Ø	60,1
150 mm Ø	147,8
Flascón 75	75
Flascón 150	150

COBAS

METABOLITES

1. **Getting ready:**

- First of all, put your samples, standards and reagents you need to **defreeze** in the bath (room temperature). After defreezing, keep your samples, standards and reagents in **ice.**
- **Fill**the "reservoir" container withmilliQwater (without) and **empty** the "waste" container in case it was full.
- **Switch on** COBAS. User: CAP, Password: RING.
- **Delete** the last data (especially important in "Routine", because COBAS will process all samples that are programmed in "Routine" when you press "Start"):
    - Go to "Routine" – F1 "Display" – F3 "Delete"
    - Go to "Info" – <2>"Patient file" – F2"Interim report" – F4 "Delete" (Sample:All (space), Test:All (space))
- Make the "**prime**" to get COBAS ready and check the needles and syringes:
    - Go to "Info" – <6> "System checks" - <1> "Prime". PressF4 "Motors off" and move the arm to the right and towards you as maximum you can. Then, select<2> "Up-sample", press F1 "Start" and check that the sample needle is releasing the liquid correctly, then press F1 "Stop". Do the same with the reagent needle: select<3> "Up-reagent", press F1 "Start" and check. Finally, select <1>"Down", press F1 "Start" and check that there are no bubbles in the syringes. If there are, go to the complete protocol. You can keep "Down" for some minutes to clean all the system (depending on how much time it was not used or which protocol was used before).
- **Set** your samples, standards and reagents: Put the standards in the little rack on the left, the reagents in the rack your test indicates, and your samples in the "Sample 1, 2, 3…" rack. It is better to program first only the standard curve. For this, just put the standards and reagents but you have to program at least one sample (it can be just water).
    - When **calculating the reagents** you need take into account: **(**(Nº of samples)+(Standards)**)***(Nº reads) + (Spare volume)
      (Spare volume is 500 µLfor"SRs"and 4 mL for"R")
- Before programming, **check the test** you will use:
    - Go to "Progr" – <2> "Tests" – F4 Test Level" - <2>"Metab" – select the letter of your "Test X".

2. **Start programming the standard curve:**

- Once we have checked that everything is in its position, there is enough water in the reservoir and enough cuvette segments, you can **program**

your reads (first only the standard curve, therefore only one sample cub with water):
    - Go to “Routine” – Select the positions (only one), press “Enter” select your test: press <2> and the letter, press “Enter” and then “Start”. (check the routine list in F1 “Display”, just in case).
- To **know** what COBAS is doing go to “Status”.
- Once the standard curve is done, you can **check** it:
    - Go to “Info” – 3 “Calibration control”– F4 “Test Level” - <2> “Metab” – select your test “Test X” – F1 “Plot”. If you have a problem with your curve go to section 5.

3. Programming your samples:

- Go to “Routine” – Select the positions (all samples you want to read first, example “1” – “to” – “10”), press “Enter” and selectyour test: press <2> and the letter, press “Enter” and then “Start”. Before starting better check the routine list: F1 “Display” and check you have enough water in the reservoir, cuvettes and reagent.

- As before, to **know** what COBAS is doing go to “Status”. You can also go to “Routine” – F1 “Display” to know if your sample is still waiting for being processed (ex: 1 GLC1), in process (ex: 1 GLC1) or already processed (ex: 1 GLC1). Once all your samples are processed, in “Status” you will read “Rack Handling Possible”. You can put your samples back in ice while COBAS is finishing with the reading of absorbance.
- To **check your results**:
    - Go to “Info” – <1>“Test results” – F1 “Data” – F3 “Raw Data” – Sample: all (enter) (to check one by one) or select one sample, then Test:<2> and the letter of your test. You can check the absorbance graphic in F1 “Plot” (especially useful when doing Lactate). If you have problems with some of your samples go to section 5.

4. Finishing COBAS

- To finish, **clean** the needles (being sure that there is enough water in the reservoir and the waste is empty):
    - Go to “Info” – <6>“System checks” – <9>“Needle cleaning”
- Finally, **empty** the waste and the reservoir (unless you have to use it the next day), **throw** the used cuvette segments (if there was Hidrazin in your reagents throw it in cytotoxic container) and **delete** your data both in “Routine” and “Info”- (2) – “Patient file” – “Interin report”.

5. Possible problems you can have

- **Standard curve:**
    - “CALC ERROR”: It means that the program cannot generate an appropriate standard curve. Check the absorbance values and consider to repeat the standard curve.

- If the standard curve is done but there is one read or one point that you prefer to eliminate: Go to “Info” - <1> “Test results” – F1 “Data” – F1 “Conv” – Sample: all (enter) – Test: (<2> and letter) and the test will appear, go ↓ until “Calibration” – F1 “Modify” – Select the standard you want to change and press the number according to the reads you want to keep (as it is indicated in the screen) or press “enter” if you want to eliminate the whole point. Then, F2 “Calc” – F1 “Calibration Plot” to check if it is better and F4 “Enter Test”.

- **Samples:**
  - “CALC RANGE”: It means that your sample is above or below the standard curve values. If it is above you can dilute your sample to measure it correctly. In both cases you can infer the concentration of your sample with the standard curve parameters (printed with the standard curve) and the absorbance (in “Info” – <1> “Test results” – F1 “Data” – F3 “Raw Data” – Sample: select your sample, then Test: <2> and the letter of your test).
  - SIGN: It means that the direction of the reaction of your sample is not the same as the reaction of your test. It can be because there was bubbles in your sample, or else the concentration of the metabolite is very low, almost 0. Be careful because the sample will keep in the routine list to be processed.

GLUCOSA 1

(hasta 12.5 mM, con regresión lineal)

LEVEL: 2 (MET)

TEST: L (GLC1)

RACK REACTIVOS:

5S3- Posición 1

Monorreactivo (Glucose HK CP, ref A11A01667 , Casa comercial ABX, cat num **305, P7**)

PATRONES: 6

Concentraciones:

0,5 mM
2,5 mM
5,0 mM
7,5 mM
10,0 mM
12,5 mM (**congelador**)

Posición: 1 a 6

Patrones internos:

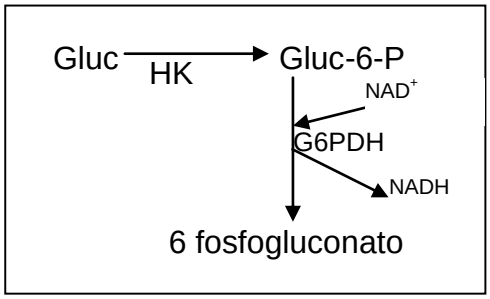
No son necesarios

PARÁMETROS RECTA REGRESIÓN:

Ro: ~ 0.00
A (pendiente): 0.0922 ± 0.003

ABS BLANCO: 0,087 ± 0,002

REACCIÓN:



COBAS MIRA 31-6807

P2 TESTS ROUTINE
GLC1 CAP /01-DEC-10

GENERAL
MEASUREMENT MODE: ABSORB
REACTION MODE : R-S
CALIBRATION MODE: LIN REGR
REAGENT BLANK : REAG/DIL
CLEANER : BEFORE

WAVELENGTH : 340 nm
DECIMAL POSITION: 3
UNIT : mmol/l

ANALYSIS
POST DIL. FACTOR: NO
CONC.FACTOR: NO

SAMPLE CYCLE: 1
VOLUME: 5.0 µl
DILUENT NAME: H2O
VOLUME: 35.0 µl

REAGENT CYCLE: 1
VOLUME: 150 µl

CALCULATION
SAMPLE LIMIT: NO

REAC. DIRECTION: INCREASE
CHECK: ON
ANTIGEN EXCESS: NO

CONVERS. FACTOR: 1.00000
OFFSET: 0.00000

TEST RANGE LOW: OFF
HIGH: OFF
NORM. RANGE LOW: NO
HIGH: NO

NUMBER OF STEPS : 1

CALC. STEP A : ENDPOINT
READINGS FIRST: CB LAST: 13

CALIBRATION
CALIB. INTERVAL: EACH RUN

BLANK
REAG. RANGE LOW: NO
HIGH: NO
BLANK RANGE LOW: NO
HIGH: NO

STANDARD POS: 1
1: 0.500 2: 2.500 mmol/l
3: 5.000 4: 7.500 mmol/l
5: 10.000 6: 12.500 mmol/l
7: NO 8: NO
REPLICATE : DUPL
DEVIATION : NO

LACTATO (3)

LEVEL: 2 (MET)

TEST: N (LAC3)

RACK REACTIVOS: 5s2, posición 4

- **R** (250 µl/muestra):

  1 mL de tampón Tris-Hidracina 0,2M EDTA 12 mM pH 9 (cámara fría)

  +

  100 µL NAD^+ 6 mg/300 µl (congelador)

- **SR1** (7 µl/muestra):

  100 µl de LDH (319, P4)

  +

  200 µL $(NH_4)_2SO_4$ 3,2 M pH 6,5 (cámara fría)

- **SR2** (10 µl/muestra): H_2O milli-Q

PATRONES
Preparados en fresco a partir de una madre de 1000 mg/L (808, P1), ver detrás:

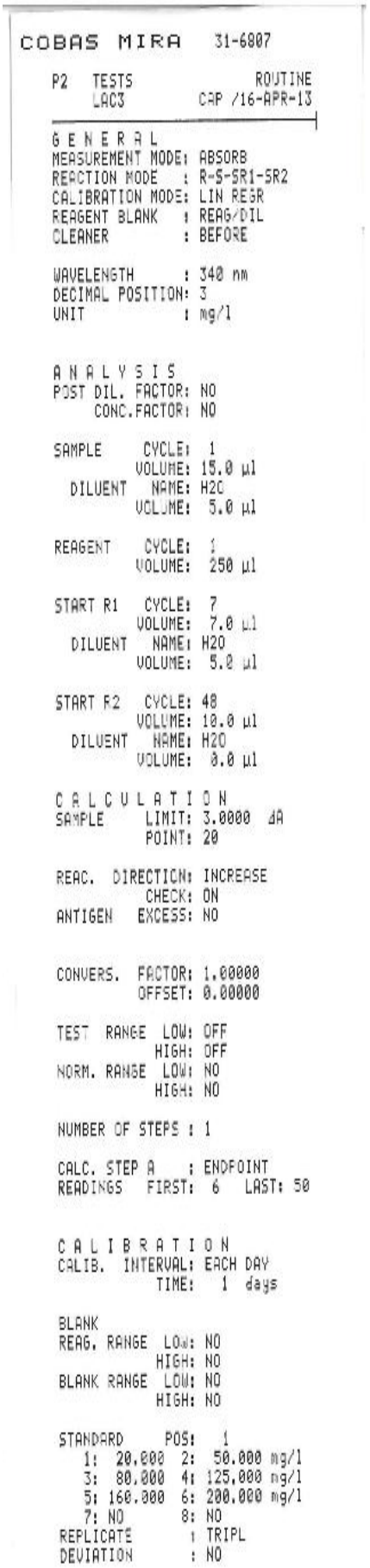
- **Concentraciones:**

20 mg/L	125 mg/L
50 mg/L	160 mg/L
80 mg/L	200 mg/L

- **Posiciones:** 1, 2, 3, 4, 5, 6.

PARÁMETROS DE LA RECTA:

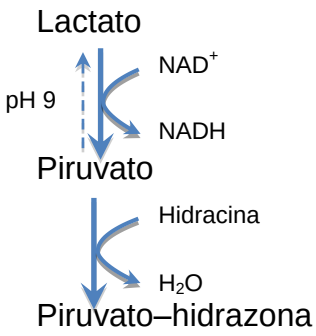
- **A =** 0,0018 ~ 0,0021
- **R_0 =** –0,02 ~ 0,02



```
COBAS MIRA   31-6807

P2  TESTS                ROUTINE
    LAC3          CAP /16-APR-13

G E N E R A L
MEASUREMENT MODE: ABSORB
REACTION MODE   : R-S-SR1-SR2
CALIBRATION MODE: LIN REGR
REAGENT BLANK   : REAG/DIL
CLEANER         : BEFORE

WAVELENGTH      : 340 nm
DECIMAL POSITION: 3
UNIT            : mg/l

A N A L Y S I S
POST DIL. FACTOR: NO
    CONC.FACTOR: NO

SAMPLE     CYCLE:  1
          VOLUME: 15.0 µl
  DILUENT   NAME: H2O
          VOLUME:  5.0 µl

REAGENT    CYCLE:  1
          VOLUME:  250 µl

START R1   CYCLE:  7
          VOLUME:  7.0 µl
  DILUENT   NAME: H2O
          VOLUME:  5.0 µl

START R2   CYCLE: 48
          VOLUME: 10.0 µl
  DILUENT   NAME: H2O
          VOLUME:  0.0 µl

C A L C U L A T I O N
SAMPLE     LIMIT: 3.0000  dA
           POINT: 20

REAC.  DIRECTION: INCREASE
          CHECK: ON
ANTIGEN  EXCESS: NO

CONVERS.  FACTOR: 1.00000
          OFFSET: 0.00000

TEST  RANGE  LOW: OFF
             HIGH: OFF
NORM. RANGE  LOW: NO
             HIGH: NO

NUMBER OF STEPS : 1

CALC. STEP A    : ENDPOINT
READINGS   FIRST:  6   LAST: 50

C A L I B R A T I O N
CALIB.  INTERVAL: EACH DAY
           TIME:   1  days

BLANK
REAG. RANGE  LOW: NO
             HIGH: NO
BLANK RANGE  LOW: NO
             HIGH: NO

STANDARD     POS:   1
   1:  20.000  2:  50.000 mg/l
   3:  80.000  4: 125.000 mg/l
   5: 160.000  6: 200.000 mg/l
   7: NO       8: NO
REPLICATE      : TRIPL
DEVIATION      : NO
```

IMPORTANTE: Comprobar siempre las curvas de absorbancia de cada una de las pinchadas para asegurar que no hay burbujas en el cubilete, y poder confirmar la concentración leída.

REACCIÓN



PREPARACIÓN DE REACTIVOS

Patrones de lactato:

	H_2O (µL)	Solución 1000 mg/L (µL)	Volumen total	Dilución	Concentración equivalente (mM)
20 mg/L	490	10	500	1/50	0,222
50 mg/L	285	15	300	1/20	0,555
80 mg/L	460	40	500	2/25	0,888
125 mg/L	350	50	400	1/8	1,388
160 mg/L	252	48	300	4/25	1,776
200 mg/L	400	100	500	1/5	2,220

Para interconvertir valores de mg/L a mM, usar la masa molecular del ácido láctico (90,08 g/mol).

Tampón hidracina 0,2M EDTA 12 mM pH 9
Para 250 mL, coger **9,92 mL de hidracina 24-26%** (419, N4) y pesar **1,12 g de Na-EDTA** (139, E5). Disolver con H_2O milli-Q, y comprobar que el pH es cercano a 9 (si no, ajustarlo un poco con HCl). Enrasar a 250 mL con más H_2O milli-Q, y comprobar que la conductividad está alrededor de 2,1 mS/cm.

GLUTAMATO

LEVEL: 2 (met)
TEST: O (GLU1)

RACK REACTIVOS:

5S3- Posición 2

- **R** (100 µl/muestra):
  10,0 ml de tampón glicina 0,5 M + hidracina 0,6 M + EDTA 2 mM, pH 9 (cámara fría) (una vez preparado usar en máximo 1 mes)
  +
  1,48 ml MIX ADP 25 mM – NAD^+ 40 mM (congelador)
- **SR1** (25 µl/muestra):
  100 µl de glutamato deshidrogenasa 1500 U/ml
  +
  1,4 ml de tampón trietanolamina (614, N4)

PATRONES:

- **Concentraciones:**

0,06 mM
0,18 mM
0,30 mM
0,45 mM
0,60 mM
0,75 mM

- **Posiciones:** de 1 a 6

PARÁMETROS DE LA RECTA:

Ro = 0,0033 ± 0,0005
A = 0,516 ± 0,008

```
COBAS MIRA    31-6807

P2   TESTS                ROUTINE
     GLU1            CAP /14-MAR-14
-------------------------------------
G E N E R A L
MEASUREMENT MODE: ABSORB
REACTION MODE   : R-S-SR1
CALIBRATION MODE: LIN REGR
REAGENT BLANK   : REAG./DIL
CLEANER         : BEFORE

WAVELENGTH      : 340 nm
DECIMAL POSITION: 2
UNIT            : mmol/l

A N A L Y S I S
POST DIL. FACTOR: NO
     CONC.FACTOR: NO

SAMPLE      CYCLE:  1
          VOLUME: 25.0 µl
  DILUENT    NAME: H2O
          VOLUME:  5.0 µl

REAGENT     CYCLE:  1
          VOLUME:  100 µl

START R1    CYCLE:  6
          VOLUME: 25.0 µl
  DILUENT    NAME: H2O
          VOLUME:  0.0 µl

C A L C U L A T I O N
SAMPLE      LIMIT: 3.0000  dA
             POINT: 20

REAC.  DIRECTION: INCREASE
            CHECK: ON
ANTIGEN   EXCESS: NO

CONVERS.   FACTOR: 1.00000
          OFFSET: 0.00000

TEST  RANGE  LOW: OFF
            HIGH: OFF
NORM. RANGE  LOW: NO
            HIGH: NO

NUMBER OF STEPS : 1

CALC. STEP A    : ENDPOINT
READINGS   FIRST:  5   LAST: 50

C A L I B R A T I O N
CALIB.  INTERVAL: EACH DAY
            TIME:   1  days

BLANK
REAG. RANGE  LOW: NO
            HIGH: NO
BLANK RANGE  LOW: NO
            HIGH: NO

STANDARD      POS:   1
   1:     0.06  2:     0.18 mmol/l
   3:     0.30  4:     0.45 mmol/l
   5:     0.60  6:     0.75 mmol/l
   7: NO        8: NO
REPLICATE       : DUPL
DEVIATION       : NO
```

DETERMINACIÓN MEDIANTE COBAS MIRA DEL GLUTAMATO DEL MEDIO DE CULTIVO

DESCRIPCIÓN DEL MÉTODO:

La determinación de glutamato y glutamina que se describe a continuación se basa en lo publicado en:

> *Lund, Patricia (1985) L-Glutamine and L-Glutamate. UV-method with Glutaminase and Glutamate Dehydrogenase. In "Methods of Enzymatic Analysis, Vol. VIII: Metabolites 3: Lipids, Amino Acids and Related Compounds" (Editor in chief: Bergmeyer, HU). Weinheim; Deerfield Beach, FL. Pp357-363.*

La determinación del glutamato se basa en la reacción:

$$\text{L-Glutamato} + NAD^+ + H_20 \rightarrow \text{2-oxoglutarato} + NADH + NH_4^+$$

La reacción la lleva a cabo la enzima glutamato deshidrogenasa.

La glutamina del medio no se mide directamente sino que se ha de transformar la glutamina en glutamato para poder determinarla. La transformación de la glutamina en glutamato se basa en la siguiente reacción:

$$\text{L-Glutamina} + H_20 + H^+ \rightarrow \text{L-glutamato} + NH_4^+$$

Esta reacción está catalizada por la enzima glutaminasa.

DETALLES PROGRAMAS, REACTIVOS Y PATRONES COBAS:

Existe un único método para medir la concentración de glutamato del medio de cultivo celular:

- **GLU:** Lee concentraciones de glutamato entre 0,05 y 0,75 mM. El método está en el Nivel 2-MET, letra O. Los patrones a utilizar son 0,06 mM, 0,18 mM, 0,30 mM, 0,45 mM 0,60 mM y 0,75 mM, los cuales se colocarán a partir de la posición 1 del rack de patrones.

En general la concentración de **glutamato** del medio de un experimento típico suele ser baja y por lo tanto medible sin necesidad de dilución previa de las muestras. En cuanto a la determinación de **glutamina** tendremos que transformar la glutamina del medio a glutamato y medir la concentración de glutamina + glutamato. Puesto que el rango de concentración de glutamina

recomendado durante la reacción con la glutaminasa es entre 0,2–0,4 mM en la mezcla de reacción es necesaria una dilución previa de dichas muestras, a razón de 1:5 o 1:10 en la solución final, dependiendo de la concentración de glutamina inicial presente en el medio.

Los reactivos del método se colocan en la **posición 2** del rack de reactivos **5s3**. Los reactivos a utilizar son:

- **R:** (100 µl/lectura)

  10,0 ml tampón Glicina 0,5 M - Hidracina 0,5 M, pH 9 (nevera)
  +
  1,48 ml MIX ADP 25 mM - NAD^+ 40 mM (congelador)

- **SR1:** (25 µl/lectura) + (volumen muerto: 500 µL)

  100µl de GLDH 1.500 U/ml (nºcat 616 (C5))
  +
  1,4 ml de tampón trietanolamina (nºcat 614 (N4), ref T0449 Sigma)

PROCEDIMIENTO EXPERIMENTAL DETALLADO:

Primero será necesario descongelar las muestras a leer junto con los patrones de glutamato y realizar un tratamiento a nuestras muestras de desproteinización para que la existencia de alguna enzima en nuestro medio de incubación no interfiera en la medida.

- **Procedimiento de desproteinización:**

- Hervir las muestras a 80ºC en bloque seco durante 15 minutos (si se hace en eppendorf recordar que es conveniente hacer un agujero en la tapa para que no estallen al calentarse). El volumen mínimo que se tiene que poner a hervir ha de ser de 500 µL.

- Poner las muestras en hielo durante 5 minutos.

- Centrifugar 10 minutos a 13.200 rpm en la microcentrífuga del laboratorio (corresponde a 16100 g).

- Recoger el sobrenadante y ponerlo en otro eppendorf (o cubilete de COBAS directamente).

- **Conversión de glutamina a glutamato**

- En función de la concentración de medio inicial y del consumo de glutamina esperado, se debe realizar una dilución final de la muestra en el medio de cultivo de aproximadamente 1:5 para 2 mM y 1:10 para 4 mM (el rango final para la reacción de la glutaminasa debe estar entre 0,2 y 0,4 mM de glutamina). Las proporciones originales del MIX glutaminasa antiguo se encuentran al final del protocolo, y hay que añadir el agua extra suficiente para diluir las muestras en la cantidad deseada:

		Dilución 1:5	Dilución 1:10
Volumen de muestra		50 µl	25 µl
Volumen de MIX		200 µl	225 µl
	Volumen H_2O*****	147,5 µl/muestra	172,5 µl/muestra
	Volumen tampón acetato	50 µl/muestra	50 µl/muestra
	Volumen glutaminasa 10 U/ml	2,5 µl/muestra	2,5 µl/muestra
Volumen total		250 µl	250 µl

**El volumen de agua es el volumen de la MIX original + el volumen de la dilución de la muestra.*

- **MUY IMPORTANTE:** Para asegurar que la reacción de la glutaminasa está funcionando correctamente se debe procesar paralelamente a las muestras un patrón de **glutamina 2,53 mM** (para dilución 1:5) **o 5,03 mM** (para dilución 1:10) y un patrón de **glutamato 1,80 mM** (para ambas diluciones), a los que se les hará el mismo tratamiento que han seguido dichas muestras.

- Para preparar la MIX Glutaminasa realizar el siguiente cálculo:

  *Volumen de MIX a preparar = (**A + 3**) * **B***,

  dónde:
  A: Nº de muestras
  B: Volumen de MIX empleado para la dilución deseada.

  Si se desea preparar otro volumen final para llevar a cabo la reacción de la glutaminasa, calcular el volumen total de MIX necesario, y aplicar la proporción de los componentes que aparece en la tabla.

- Dejar incubar durante 30min (a ser posible no más de 40 min) a 37ºC con agitación. Al acabar la incubación vortear y congelar o poner las

muestras en frío para parar la reacción y evitar la degradación del glutamato formado.

- **Lectura de concentración de glutamato**

- Mientras se lleva a cabo la conversión de glutamina a glutamato se realizará la lectura de glutamato en el medio desproteinizado sobrante.

- **Lectura de concentración de glutamato + glutamina**

- Una vez pasado el tiempo necesario para llevar a cabo la reacción de transformación de la glutamina a glutamato procederemos a la lectura de la concentración glutamina + glutamato con el método GLU como se ha explicado anteriormente. En esta determinación el valor de concentración que nos de el Cobas se tendrá que multiplicar por el factor de dilución utilizado a la hora de la reacción con la glutaminasa.

- Una vez hechas las correspondientes lecturas para determinar la concentración de glutamina se le restará a la concentración glutamina + glutamato la concentración de glutamato leída en el medio desproteinizado.

PREPARACIÓN DE REACTIVOS

Determinación de glutamato en cobas

1.- Tampón Glicina 0,5 M – Hidracina 0,6 M – EDTA 2 mM, pH 9 (1 mes a 4ºC)

Para 100ml: Disolver 3,75 g de glicina (nºcat 812 (E11), ref A3707 Applichem), + 12,4 ml de hidracina Dihidroclorada (·2HCl) líquida (nºcat 419 (CF), ref 53847 Fluka) y 0,075 g de EDTA (nº cat 139 (E5)) en aproximadamente 75ml de agua Milli-Q. Ajustar el pH a 9 con HCl concentrado (37%), traspasar a matraz aforado/probeta de 100ml y enrasar con agua Milli-Q.

2.- Mix ADP 25 mM - NAD^+ 40 mM (-20ºC)

Para 25ml: Disolver 0,265 g de ADP (nºcat 69, (C1) ref A2754 Sigma) y 0,6634g de NAD^+ (nºcat 496, (P4) ref 1012799.0001 Roche) en aproximadamente 15ml de agua Milli-Q, traspasar a matraz aforado de 25ml y enrasar con agua Milli-Q.

3.- Glutamato deshidrogenasa (GLDH) 1.500 U/ml (-20ºC)

El reactivo comercial GLDH 3000U liofilizado (ref. 197737 Roche) se resuspende con 2 ml de agua Milli-Q. Se alícuota en unidades de 100 µL y se guardan congeladas.

Transformación de glutamina a glutamato por la reacción con glutaminasa

4.- Tampón acetato 0,5 M, pH 5,0

4.1.- Preparar solución **(A) Acetato de sodio 0,5 M:** pesar 6,8 g de Acetato de Sodio (CH_3COONa) (nºcat 101 (E5), ref 131633 Panreac), disolverlo en aproximadamente 50ml de agua Milli-Q, traspasar a matraz aforado de 100ml y enrasar con agua Milli-Q.

4.2.- Preparar solución **(B) Ácido acético 0,5 M:** pipetear 2,9 ml de ácido acético (CH_3COOH) (nºcat 383 (CAA), ref A-6283 Sigma), colocarlo en un matraz aforado de 100ml y enrasarlo con agua Milli-Q .

4.3.- Mezclar 67,8 ml **A** con 32.2 ml **B**; comprobar el pH. Ajustar el pH hasta 5,0 añadiendo el volumen adecuado de solución **A** (para basificar o aumentar el pH) o solución **B** (para acidificar o disminuir el pH).

5.- Glutaminasa 10 U/ml:

El reactivo comercial glutaminasa 50 U (nºcat 497 (C1), ref G-8880 Sigma) se resuspende con 5 ml de tampón acetato 5 mM, pH 6,0 (o si el envase es de 10 U se resuspende con 1 ml del mismo tampón). Se alícuota en unidades de 200 µL y se guardan congeladas. Comprobar siempre las condiciones de redisolución en la hoja técnica del fabricante.

6.- Tampón acetato 5 mM, pH 6,0 (para preparar *Glutaminasa 10 U/ml*)**:**

Se hace dilución 1:100 del tampón acetato 0,5 M, pH 5,0 y se reajusta el pH hasta 6,0 usando una solución de NaOH.

CÉLULAS PROLIFERANTES:

Asumiendo **crecimiento exponencial** del número de células y **consumo o producción proporcional al número de células y con la misma constante durante todo el experimento**, las fórmulas matemáticas que permiten obtener la tasa de crecimiento **μ**[10] y la tasa de producción o consumo **k_{pc}** serían las siguientes:

$$\mu = \frac{\ln\left(\frac{N_f}{N_0}\right)}{t_f} \qquad y \qquad k_{PC} = \frac{\Delta M}{\Delta N} \times \mu \times 10^9$$

Con unidades:

$\mu \rightarrow h^{-1}$
$k_{PC} \rightarrow$ µmol/(million cells · h)

donde,

> **$\Delta N = N_f - N_o$**, es el incremento de células producido durante un tiempo **$t = t_f$**, con **N_o** como la cantidad de células inicial y **N_f** como la cantidad de células final, siendo **N_f/N_o** la proliferación.
> **$\Delta M = M_f - M_o$**, es la cantidad de metabolito consumido o producido durante un tiempo **$t = t_f$**, con **M_o** como la cantidad de metabolito inicial y **M_f** como la cantidad de metabolito final.

por ejemplo, dado un consumo de glucosa de 1.92 mM en un volumen de 10 ml en un tiempo de 72 horas y donde el numero inicial de células fuera 0.5×10^6 y el número final de 10^6.

queda, **ΔM** = 1.92 mM × 10 ml = 0.0192 mmol
ΔN = 0.5×10^6 cell
N_f/N_o = 2
t_f = 72 h

$$\mu = 0.0095 h^{-1} \qquad y \qquad k_{PC} = 0.37 \frac{\mu mol}{million cell \cdot h}$$

Deducción:

[10] Cuando el valor de µ es negativo se habla de "tasa de muerte celular" en lugar de crecimiento

La fórmula de arriba se deduce a partir del siguiente sistema de ecuaciones diferenciales:

$$\begin{cases} dN_t/dt = N_t \times \mu \\ dM_t/dt = N_t \times k_{PC} \end{cases}$$

La primera ecuación diferencial describe **crecimiento exponencial** a una tasa de crecimiento **μ** en el tiempo **t**. La segunda ecuación diferencial describe **consumo o producción proporcional al número de células** en el tiempo **t**. Este sistema se puede solucionar a un tiempo $\mathbf{t_f}$ tomando como valores iniciales $\mathbf{N_o}$ y $\mathbf{M_o}$. Dado que hay crecimiento, entonces $\mathbf{N_o < N_f}$ y la solución quedará como:

$$N_f = N_0 \times e^{\mu \times t_f}$$

$$M_f = \frac{\mu \times M_0 - k_{PC} \times N_0 + e^{\mu \times t_f} \times k_{PC} \times N_0}{\mu}$$

de donde se deducen la fórmulas de arriba para **μ** y $\mathbf{k_{pc}}$.

CÉLULAS PROLIFERANTES CON INHIBIDOR DE LA PROLIFERACIÓN:

Cuando las células se tratan con un inhibidor de la proliferación/viabilidad celular, si se considera que el efecto del inhibidor es constante en todo el tiempo de tratamiento, la tasa de proliferación celular ***μ*** se ve modificada por una constante de toxicidad $\boldsymbol{k_T}$:

$$\mu = \mu_{\max} \times (1 - k_T)$$

donde:

- ***μ***, es la tasa de proliferación celular de las células tratadas
- $\boldsymbol{\mu_{max}}$, es la tasa de proliferación celular máxima, correspondiente a las células control sin tratar con ningún inhibidor de la proliferación celular
- $\boldsymbol{k_T}$, es la tasa de inhibición de la proliferación celular

Teniendo en cuenta esto, el valor de $\boldsymbol{\mu_{max}}$ correspondería al valor de ***μ*** obtenido en las células sin tratar, y los valores de $\boldsymbol{k_T}$ y $\boldsymbol{k_{PC}}$ de unas células tratadas serían, tras resolver el sistema de ecuaciones diferenciales:

$$\mu = \mu_{\max} \times (1 - k_T) = \frac{\ln\left(\frac{N_f}{N_0}\right)}{t_f}$$

$$k_T = 1 - \frac{\mu}{\mu_{\max}} \quad \text{o} \quad k_T = 1 - \frac{\ln\left(N_f / N_0\right)}{\mu_{\max} \cdot t_f}$$

$$k_{PC} = \frac{\Delta M}{\Delta N} \times \mu \times 10^9 \quad \text{o} \quad k_{PC} = \frac{\Delta M}{\Delta N} \times \mu_{\max} \times (1 - k_T) \times 10^9$$

con unidades:

$k_T \rightarrow$ sin unidades
$\boldsymbol{k_{PC}} \rightarrow$ µmol/(million cells · h)

CÉLULAS NO PROLIFERANTES:

Obsérvese que sin proliferación ($\mathbf{N_o = N_f}$) el sistema de ecuaciones diferenciales quedaría como:

$$\begin{cases} dN_t / dt = 0 \\ dM_t / dt = N_t \times k_{PC} \end{cases}$$

y su solución como:

$$M_f = M_0 + N_0 \times k_{PC} \times t_f$$

de donde se deduce,

$$k_{PC} = \frac{\Delta M}{N_0 \times t_f} \times 10^9$$

con unidades:

$\boldsymbol{k_{PC}} \rightarrow$ µmol/(million cells · h)

CÉLULAS NO PROLIFERANTES CON INHIBIDOR DE LA PROLIFERACIÓN:

En este caso, $\mathbf{N_o > N_f}$ y el sistema de ecuaciones diferenciales quedaría como:

$$\begin{cases} dN_t/dt = -N_t \times k_T \\ dM_t/dt = N_t \times k_{PC} \end{cases}$$

y su solución como:

$$k_T = \frac{\ln\left(N_0/N_f\right)}{t_t}$$

$$M_f = \frac{M_0 \times k_T + N_0 \times k_{PC} - Nf \times k_{PC}}{k_T}$$

De donde se deduce:

$$k_{PC} = -\frac{\Delta M}{\Delta N} \times k_T \times 10^9$$

con unidades:

k_{PC} → µmol/(million cells · h)

ANNEX V: COBAS Results

Here there are the photocopied results printed by the spectrophotometer after each measurement. Those result are all in the excel calculation sheets.

Those two are mistakes that I could gather to show how the machine prints them.

COBAS MIRA 31-6807

BIOQUIMICA INTEGRATIVA

TEST RESULTS

GLC1 BLANK -0.0020

* Ro 0.00531
* A 0.09131

STD-1	0.50 mmol/l	<CALC RANGE
STD11	0.50 mmol/l	<CALC RANGE
STD21	0.50 mmol/l	<CALC RANGE
STD-2	2.51 mmol/l	
STD12	2.52 mmol/l	
STD22	2.50 mmol/l	
STD-3	5.05 mmol/l	
STD13	5.04 mmol/l	
STD23	5.06 mmol/l	
STD-4	7.67 mmol/l	
STD14	7.59 mmol/l	
STD24	7.75 mmol/l	
STD-5	9.66 mmol/l	
STD15	9.59 mmol/l	
STD25	9.72 mmol/l	
STD-6	12.50 mmol/l	>CALC RANGE
STD16	12.50 mmol/l	>CALC RANGE
STD26	12.50 mmol/l	>CALC RANGE

61 SIGN

CAP 18-JUL-17 / 11:53

COBAS MIRA 31-6807

BIOQUIMICA INTEGRATIVA

TEST RESULTS

1/2 (more)

GLC1	1	6.81 mmol/l
		6.83 mmol/l
	2	6.51 mmol/l
		6.50 mmol/l
	3	6.45 mmol/l
		6.51 mmol/l
	4	6.26 mmol/l
		6.26 mmol/l
	5	6.25 mmol/l
		6.41 mmol/l
	6	6.35 mmol/l
		6.47 mmol/l
	7	6.34 mmol/l
		6.31 mmol/l
	8	6.44 mmol/l
		6.48 mmol/l
	9	6.19 mmol/l
		6.18 mmol/l
	10	6.04 mmol/l
		6.00 mmol/l
	11	6.04 mmol/l
		6.08 mmol/l
	12	6.20 mmol/l
		6.30 mmol/l
	13	6.05 mmol/l
		6.17 mmol/l
	14	6.19 mmol/l
		6.29 mmol/l
	19	5.75 mmol/l
		5.89 mmol/l
	20	5.92 mmol/l
		5.87 mmol/l

GLC1	1	>12.50 mmol/l	
	2	>12.50 mmol/l	
	3	>12.50 mmol/l	
	4	12.50 mmol/l	>CALC RANGE
	5	>12.50 mmol/l	
	6	>12.50 mmol/l	
	7	>12.50 mmol/l	
	8	>12.50 mmol/l	
	9	12.50 mmol/l	>CALC RANGE
	10	12.50 mmol/l	>CALC RANGE
	11	>12.50 mmol/l	
	12	12.50 mmol/l	>CALC RANGE
	13	>12.50 mmol/l	
	14	>12.50 mmol/l	
	15	12.21 mmol/l	
	16	11.97 mmol/l	
	17	12.12 mmol/l	
	18	12.33 mmol/l	
	19	12.50 mmol/l	>CALC RANGE
	20	12.50 mmol/l	>CALC RANGE
	21	12.02 mmol/l	
	22	11.92 mmol/l	
	23	11.98 mmol/l	
	24	12.43 mmol/l	
	25	12.40 mmol/l	
	26	12.06 mmol/l	
	27	9.81 mmol/l	
	28	9.38 mmol/l	
	29	9.58 mmol/l	
	30	9.70 mmol/l	

IP 18-JUL-17 / 12:20

TEST RESULTS

GLC1 BLANK -0.0020

* Ro 0.00531
* A 0.09131

STD-1	0.50	mmol/l	<CALC RANG
STD11	0.50	mmol/l	<CALC RANG
STD21	0.50	mmol/l	<CALC RANG
STD-2	2.51	mmol/l	
STD12	2.52	mmol/l	
STD22	2.50	mmol/l	
STD-3	5.05	mmol/l	
STD13	5.04	mmol/l	
STD23	5.06	mmol/l	
STD-4	7.67	mmol/l	
STD14	7.59	mmol/l	
STD24	7.75	mmol/l	
STD-5	9.66	mmol/l	
STD15	9.59	mmol/l	
STD25	9.72	mmol/l	
STD-6	12.50	mmol/l	>CALC RANG
STD16	12.50	mmol/l	>CALC RANG
STD26	12.50	mmol/l	>CALC RANG

61

SIGN

CAP 18-JUL-17 / 11:

COBAS MIRA 31-6807

BIOQUIMICA INTEGRATIVA

TEST RESULTS

C1	31	9.32	mmol/l
	32	9.74	mmol/l
	33	8.04	mmol/l
	34	7.92	mmol/l
	35	7.79	mmol/l
	36	8.50	mmol/l
	37	8.22	mmol/l
	38	8.24	mmol/l

COBAS MIRA 31-6807

BIOQUIMICA INTEGRATIVA

TEST RESULTS

.C1	31	9.52	mmol/l
	32	9.84	mmol/l
	33	8.15	mmol/l
	34	8.04	mmol/l
	35	8.21	mmol/l
	36	8.40	mmol/l
	37	8.42	mmol/l
	38	8.25	mmol/l

COBAS MIRA 31-6807

BIOQUIMICA INTEGRATIVA

TEST RESULTS

GLC1	15	12.01	mmol/l
	16	11.89	mmol/l
	17	12.16	mmol/l
	18	12.20	mmol/l
	20	12.23	mmol/l
	21	11.81	mmol/l
	22	11.45	mmol/l
	23	11.82	mmol/l
	24	11.94	mmol/l
	25	11.79	mmol/l
	26	11.71	mmol/l
	27	9.55	mmol/l
	28	9.16	mmol/l
	29	9.52	mmol/l
	30	9.78	mmol/l

CAP 18-JUL-17 / 13:30

TEST RESULTS

LAC3	1	0.505	mmol/l	R
	2	0.472	mmol/l	R
	3	0.361	mmol/l	R
	4	0.222	mmol/l	R <CALC RANG
	5	0.337	mmol/l	R
	6	0.222	mmol/l	R <CALC RANG
x	7	0.437	mmol/l	R
	8	0.334	mmol/l	R
	9	0.376	mmol/l	R
	10	0.435	mmol/l	R
	11	0.388	mmol/l	R
	12	0.379	mmol/l	R
	13	0.393	mmol/l	R
	14	0.371	mmol/l	R
	15	0.516	mmol/l	R
x	16	0.474	mmol/l	R
	17	0.493	mmol/l	R
	18	0.520	mmol/l	R
	19	0.505	mmol/l	R
~	20	1.204	mmol/l	R
	21	0.729	mmol/l	R
	22	0.520	mmol/l	R
	23	0.588	mmol/l	R
x	24	1.231	mmol/l	R
~	25	0.954	mmol/l	R
	26	0.535	mmol/l	R
~	27	0.973	mmol/l	R
~	28	1.107	mmol/l	R
	29	0.945	mmol/l	R
	30	0.915	mmol/l	R

COBAS MIRA 31-6807

BIOQUIMICA INTEGRATIVA

TEST RESULTS

LAC3	~ 31	1.000	mmol/l	R
	~ 32	0.972	mmol/l	R
	x 33	1.145	mmol/l	R
	x 34	1.214	mmol/l	R
	35	1.189	mmol/l	R
	36	1.076	mmol/l	R
	~ 37	1.153	mmol/l	R
	x 38	1.291	mmol/l	R
	~ 39	0.891	mmol/l	R
	x 40	1.589	mmol/l	R
	41	0.644	mmol/l	R
	42	0.765	mmol/l	R
	43	0.647	mmol/l	R
	x 44	0.917	mmol/l	R
	45	0.934	mmol/l	R
	~ 46	0.945	mmol/l	R
	47	1.194	mmol/l	R
	48	1.215	mmol/l	R
~	~ 49	1.188	mmol/l	R
~	50	1.029	mmol/l	R
	51	1.039	mmol/l	R
	52	1.053	mmol/l	R
	53	1.268	mmol/l	R
	54	1.514	mmol/l	R
	55	1.609	mmol/l	R
x	56	1.974	mmol/l	R

COBAS MIRA 31-6807

BIOQUIMICA INTEGRATIVA

TEST RESULTS
CONVERTED

LAC3	BLANK	0.0005		R
	* Ro-0.02273			
	* A 0.17760			
	STD-1	0.321	mmol/l	R
	STD11	0.487	mmol/l	D
	STD21	0.408	mmol/l	D
	STD31	0.321	mmol/l	R
	STD-2	0.382	mmol/l	R
	STD12	0.222	mmol/l	D <CALC RANGE
	STD22	0.382	mmol/l	R
	STD32	0.222	mmol/l	D <CALC RANGE
	STD-3	0.932	mmol/l	R
	STD13	0.923	mmol/l	R
	STD23	0.937	mmol/l	R
	STD33	0.937	mmol/l	R
	STD-4	1.425	mmol/l	R
	STD14	1.417	mmol/l	R
	STD24	1.460	mmol/l	R
	STD34	1.399	mmol/l	R
	STD-5	1.779	mmol/l	R
	STD15	1.742	mmol/l	R
	STD25	1.752	mmol/l	R
	STD35	1.842	mmol/l	R
	STD-6	2.210	mmol/l	R
	STD16	2.161	mmol/l	R
	STD26	2.148	mmol/l	R
	STD36	2.220	mmol/l	R >CALC RANGE
	61	<0.222	mmol/l	R

CAP 19-JUL-17 / 12:0

TEST RESULTS

LAC3	31	0.922	mmol/l	R
	32	0.899	mmol/l	R
	33	1.166	mmol/l	R
~	34	1.196	mmol/l	R
	35	1.187	mmol/l	R
	36	1.078	mmol/l	R
	37	1.046	mmol/l	R
~	38	1.238	mmol/l	R
	39	0.930	mmol/l	R
	40	1.615	mmol/l	R
	4	0.520	mmol/l	R
		0.514	mmol/l	R
	6	0.504	mmol/l	R
		0.500	mmol/l	R
	20	0.587	mmol/l	R
	25	0.509	mmol/l	R
	28	0.933	mmol/l	R
	41	0.645	mmol/l	R
~	42	0.660	mmol/l	R
	43	0.654	mmol/l	R
	44	0.935	mmol/l	R
	45	0.935	mmol/l	R
~	46	0.994	mmol/l	R
~	47	1.194	mmol/l	R
	48	1.202	mmol/l	R
	49	1.198	mmol/l	R
	50	1.030	mmol/l	R
	51	1.054	mmol/l	R
	52	1.050	mmol/l	R
	53	1.220	mmol/l	R
	54	1.470	mmol/l	R
	55	1.636	mmol/l	R
	56	1.978	mmol/l	R
~	57	2.179	mmol/l	R
	58	2.143	mmol/l	R

TEST RESULTS

:3	1	0.499	mmol/l	R
	2	0.480	mmol/l	R
x	3	0.222	mmol/l	R <CALC RANGE
	4	0.323	mmol/l	R
	5	0.340	mmol/l	R
	6	0.222	mmol/l	R <CALC RANGE
	7	0.324	mmol/l	R
X	8	0.464	mmol/l	R
	9	0.377	mmol/l	R
X	10	0.430	mmol/l	R
	11	0.397	mmol/l	R
x	12	0.385	mmol/l	R
	13	0.389	mmol/l	R
	14	0.389	mmol/l	R
	15	0.513	mmol/l	R
	16	0.499	mmol/l	R
X	17	0.601	mmol/l	R
x	18	0.489	mmol/l	R
x	19	0.538	mmol/l	R
x	20	0.460	mmol/l	R
~	21	0.492	mmol/l	R
	22	0.523	mmol/l	R
	23	0.554	mmol/l	R
	24	0.514	mmol/l	R
	25	0.558	mmol/l	R
	26	0.628	mmol/l	R
	27	0.905	mmol/l	R
	28	0.915	mmol/l	R
	29	0.903	mmol/l	R
	30	0.918	mmol/l	R

COBAS MIRA 31-6807

BIOQUIMICA INTEGRATIVA

TEST RESULTS
CONVERTED

LAC3 BLANK 0.0005 R

* Ro-0.02273
* A 0.17760

STD-1	0.321	mmol/l	R
STD11	0.487	mmol/l	D
STD21	0.408	mmol/l	D
STD31	0.321	mmol/l	R
STD-2	0.382	mmol/l	R
STD12	0.222	mmol/l	D <CALC RANGE
STD22	0.382	mmol/l	R
STD32	0.222	mmol/l	D <CALC RANGE
STD-3	0.932	mmol/l	R
STD13	0.923	mmol/l	R
STD23	0.937	mmol/l	R
STD33	0.937	mmol/l	R
STD-4	1.425	mmol/l	R
STD14	1.417	mmol/l	R
STD24	1.460	mmol/l	R
STD34	1.399	mmol/l	R
STD-5	1.779	mmol/l	R
STD15	1.742	mmol/l	R
STD25	1.752	mmol/l	R
STD35	1.842	mmol/l	R
STD-6	2.210	mmol/l	R
STD16	2.161	mmol/l	R
STD26	2.148	mmol/l	R
STD36	2.220	mmol/l	R >CALC RANGE
61	<0.222	mmol/l	R

CAP 19-JUL-17 / 12:0

COBAS MIRA 31-6807

BIOQUIMICA INTEGRATIVA

TEST RESULTS

LAC3	X 34	1.184	mmol/l	R
	38	1.154	mmol/l	R
	46	0.979	mmol/l	R
~	57	2.204	mmol/l	R

BIOQUIMICA INTEGRATIVA

TEST RESULTS

LAC3	31	0.922	mmol/l	R	
	32	0.899	mmol/l	R	
	33	1.166	mmol/l	R	
→	~34	1.196	mmol/l	R	
	35	1.187	mmol/l	R	
	36	1.078	mmol/l	R	
	37	1.046	mmol/l	R	
→	~38	1.238	mmol/l	R	
	39	0.930	mmol/l	R	
	40	1.615	mmol/l	R	
	4	0.520	mmol/l	R	
		0.514	mmol/l	R	
	6	0.504	mmol/l	R	
		0.500	mmol/l	R	
	20	0.587	mmol/l	R	
	25	0.509	mmol/l	R	
	28	0.933	mmol/l	R	
	41	0.645	mmol/l	R	
	~42	0.660	mmol/l	R	
	43	0.654	mmol/l	R	
	44	0.935	mmol/l	R	
	45	0.935	mmol/l	R	
→	~46	0.994	mmol/l	R	
	~47	1.194	mmol/l	R	
	48	1.202	mmol/l	R	
	49	1.198	mmol/l	R	
	50	1.030	mmol/l	R	
	51	1.054	mmol/l	R	
	52	1.050	mmol/l	R	
	53	1.220	mmol/l	R	
	54	1.470	mmol/l	R	
	55	1.636	mmol/l	R	
	56	1.978	mmol/l	R	
→	~57	2.179	mmol/l	R	
	58	2.143	mmol/l	R	

COBAS MIRA 31-6807

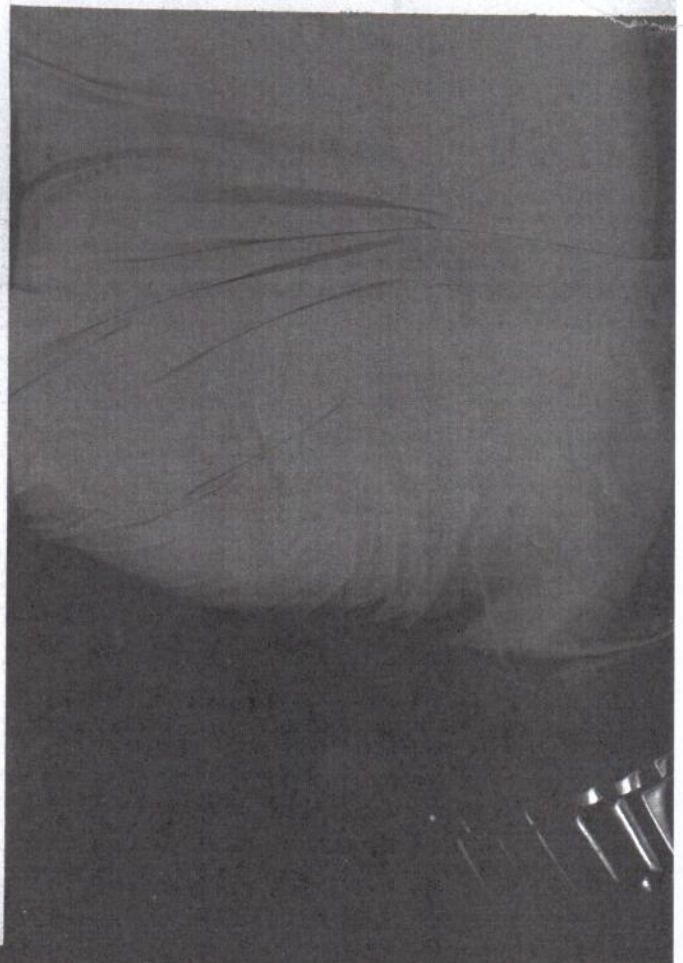
BIOQUIMICA INTEGRATIVA

TEST RESULTS

LAC3	1	0.505	mmol/l	R	
	2	0.472	mmol/l	R	
	3	0.361	mmol/l	R	
	~4	0.222	mmol/l	R	<CALC
	5	0.337	mmol/l	R	
	~6	0.222	mmol/l	R	<CALC
	x7	0.437	mmol/l	R	
	8	0.334	mmol/l	R	
	9	0.376	mmol/l	R	
	10	0.435	mmol/l	R	
	11	0.388	mmol/l	R	
	12	0.379	mmol/l	R	
	13	0.393	mmol/l	R	
	14	0.371	mmol/l	R	
	15	0.516	mmol/l	R	
	x16	0.474	mmol/l	R	
	17	0.493	mmol/l	R	
	18	0.520	mmol/l	R	
	19	0.505	mmol/l	R	
	~20	1.204	mmol/l	R	
	21	0.729	mmol/l	R	
	22	0.520	mmol/l	R	
	23	0.588	mmol/l	R	
	x24	1.231	mmol/l	R	
	~25	0.954	mmol/l	R	
	26	0.535	mmol/l	R	
	27	0.973	mmol/l	R	
	~28	1.107	mmol/l	R	
	29	0.945	mmol/l	R	
	30	0.915	mmol/l	R	

TEST RESULTS

LAC3	~31	1.000	mmol/l	R	
	~32	0.972	mmol/l	R	
	x33	1.145	mmol/l	R	
	x34	1.214	mmol/l	R	
	35	1.189	mmol/l	R	
	36	1.076	mmol/l	R	
	~37	1.153	mmol/l	R	
	x38	1.291	mmol/l	R	
	~39	0.891	mmol/l	R	
	x40	1.589	mmol/l	R	
	41	0.644	mmol/l	R	
	42	0.765	mmol/l	R	
	43	0.647	mmol/l	R	
	x44	0.917	mmol/l	R	
	45	0.934	mmol/l	R	
	~46	0.945	mmol/l	R	
	47	1.194	mmol/l	R	
	48	1.215	mmol/l	R	
	~~49	1.188	mmol/l	R	
	~50	1.029	mmol/l	R	
	51	1.039	mmol/l	R	
	52	1.053	mmol/l	R	
	53	1.268	mmol/l	R	
	54	1.514	mmol/l	R	
	55	1.609	mmol/l	R	
	x56	1.974	mmol/l	R	
	x57	2.205	mmol/l	R	
	x58	2.126	mmol/l	R	



COBAS MIRA 31-6807

BIOQUIMICA INTEGRATIVA

TEST RESULTS

Glutamat I

GLU1	1	0.130 mmol/l	→ M.I. C
	2	0.093 mmol/l	→ M.I. βOH
	3	0.096 mmol/l	480 24h C
	4	0.100 mmol/l	
	5	0.094 mmol/l	
	6	0.096 mmol/l	480 24h βOH
	7	0.097 mmol/l	
	8	0.099 mmol/l	
	9	0.129 mmol/l	480 48h C
	10	0.126 mmol/l	
	11	0.131 mmol/l	
	12	0.132 mmol/l	480 48h βOH
	13	0.135 mmol/l	
	14	0.139 mmol/l	
	15	0.153 mmol/l	LiM2 24h C
	16	0.114 mmol/l	
	17	0.119 mmol/l	
620 24h βOH.2 ←	19	0.110 mmol/l	~~→ 620 24h βOH 2~~
	18	0.125 mmol/l	~~→ 620 24h βOH 3~~
620 24h βOH 3 ←	20	0.115 mmol/l	→ LiM2 24h βOH 1
	21	0.130 mmol/l	480 48h C
	22	0.129 mmol/l	
	23	0.125 mmol/l	
	24	0.141 mmol/l	480 48h βOH
	25	0.140 mmol/l	
	26	0.138 mmol/l	
	27	0.182 mmol/l	620 48h C
	28	0.195 mmol/l	
	29	0.181 mmol/l	
	30	0.227 mmol/l	620 48h βOH
	31	0.186 mmol/l	
	32	0.193 mmol/l	
	33	0.200 mmol/l	LiM2 48h C
	34	0.218 mmol/l	
	35	0.208 mmol/l	
	36	0.209 mmol/l	LiM2 48h βOH
	37	0.194 mmol/l	
	38	0.216 mmol/l	
	39	0.106 mmol/l	620 24h C
	40	0.136 mmol/l	
	41	0.106 mmol/l	
	42	0.110 mmol/l	620 24h βOH
	43	0.107 mmol/l	
	44	0.113 mmol/l	
	45	0.118 mmol/l	→ LiM2 24h βOH 2
	46	0.117 mmol/l	→ LiM2 24h βOH 3

CAP 20-JUL-17 / 12:59

OBAS MIRA 31-6807

BIOQUIMICA INTEGRATIVA

TEST RESULTS

1 BLANK -0.0009

* Ro 0.00390
* A 0.49792

STD-1	0.060 mmol/l	<CALC RANGE
STD11	0.060 mmol/l	<CALC RANGE
STD21	0.060 mmol/l	<CALC RANGE
STD31	0.060 mmol/l	<CALC RANGE
STD-2	0.175 mmol/l	
STD12	0.175 mmol/l	
STD22	0.177 mmol/l	
STD32	0.175 mmol/l	
STD-3	0.315 mmol/l	
STD13	0.298 mmol/l	
STD23	0.314 mmol/l	
STD33	0.334 mmol/l	
STD-4	0.451 mmol/l	
STD14	0.460 mmol/l	
STD24	0.443 mmol/l	
STD34	0.450 mmol/l	
STD-5	0.601 mmol/l	
STD15	0.601 mmol/l	
STD25	0.600 mmol/l	
STD35	0.603 mmol/l	
STD-6	0.744 mmol/l	
STD16	0.744 mmol/l	
STD26	0.750 mmol/l	>CALC RANGE
STD36	0.742 mmol/l	

91 SIGN

20-JUL-17 / 11:52

COBAS MIRA 31-6807

BIOQUIMICA INTEGRATIVA

TEST RESULTS

Glutamat II

GLU1	1	0.097	mmol/l
	2	0.090	mmol/l
	3	0.092	mmol/l
	4	0.088	mmol/l
	5	0.093	mmol/l
	6	0.094	mmol/l
	7	0.110	mmol/l
	8	0.096	mmol/l
	9	0.123	mmol/l
	10	0.126	mmol/l
	11	0.128	mmol/l
	12	0.129	mmol/l
	13	0.130	mmol/l
	14	0.135	mmol/l
	15	0.111	mmol/l
	16	0.112	mmol/l
	17	0.117	mmol/l
	18	0.118	mmol/l
	27	0.209	mmol/l
	28	0.184	mmol/l
	29	0.183	mmol/l
	30	0.183	mmol/l
	31	0.188	mmol/l
	32	0.196	mmol/l
	33	0.202	mmol/l
	34	0.199	mmol/l
	35	0.197	mmol/l
	→ 37	0.196	mmol/l
GLU1	→ 36	0.198	mmol/l
	38	0.222	mmol/l
	39	0.107	mmol/l
	40	0.101	mmol/l
	41	0.110	mmol/l
	42	0.110	mmol/l
	43	0.143	mmol/l
	44	0.140	mmol/l
	45	0.118	mmol/l
	46	0.119	mmol/l

CAP 20-JUL-17 / 16:46

BAS MIRA 31-6807

IOQUIMICA INTEGRATIVA

TEST RESULTS

BLANK -0.0009

* Ro 0.00390
* A 0.49792

STD-1	0.060	mmol/l	<CALC RANGE
STD11	0.060	mmol/l	<CALC RANGE
STD21	0.060	mmol/l	<CALC RANGE
STD31	0.060	mmol/l	<CALC RANGE
STD-2	0.175	mmol/l	
STD12	0.175	mmol/l	
STD22	0.177	mmol/l	
STD32	0.175	mmol/l	
STD-3	0.315	mmol/l	
STD13	0.298	mmol/l	
STD23	0.314	mmol/l	
STD33	0.334	mmol/l	
STD-4	0.451	mmol/l	
STD14	0.460	mmol/l	
STD24	0.443	mmol/l	
STD34	0.450	mmol/l	
STD-5	0.601	mmol/l	
STD15	0.601	mmol/l	
STD25	0.600	mmol/l	
STD35	0.603	mmol/l	
STD-6	0.744	mmol/l	
STD16	0.744	mmol/l	
STD26	0.750	mmol/l	>CALC RANGE
STD36	0.742	mmol/l	

91 SIGN

P 20-JUL-17 / 11:5

COBAS MIRA 31-6807

BIOQUIMICA INTEGRATIVA

TEST RESULTS

Glutamina II

GLU1	47	0.623	mmol/l
	48	0.631	mmol/l
	49	0.623	mmol/l
	50	0.596	mmol/l
	51	0.615	mmol/l
	52	0.590	mmol/l
	53	0.591	mmol/l
	54	0.587	mmol/l
	55	0.586	mmol/l
	56	0.576	mmol/l
	57	0.594	mmol/l
	58	0.604	mmol/l
	59	0.590	mmol/l
	60	0.577	mmol/l
	43	0.105	mmol/l
	44	0.108	mmol/l
	61	0.584	mmol/l
		0.579	mmol/l
	62	0.557	mmol/l
	63	0.589	mmol/l
	64	0.572	mmol/l
		0.574	mmol/l
	65	0.524	mmol/l
	66	0.591	mmol/l
	67	0.481	mmol/l
	68	0.487	mmol/l
	69	0.484	mmol/l
	70	0.483	mmol/l
	71	0.472	mmol/l
	72	0.481	mmol/l
	73	0.438	mmol/l
	74	0.435	mmol/l
	75	0.447	mmol/l
	76	0.500	mmol/l
	77	0.436	mmol/l
	78	0.440	mmol/l
	79	0.406	mmol/l
	80	0.394	mmol/l
	81	0.388	mmol/l
	82	0.406	mmol/l
		0.401	mmol/l
	83	0.386	mmol/l
	84	0.393	mmol/l
	85	0.396	mmol/l
	86	0.535	mmol/l
	87	>0.750	mmol/l

CAP

20-JUL-17 / 18:22

AS MIRA 31-6807

QUIMICA INTEGRATIVA

EST RESULTS

tamina I

	47	0.629	mmol/l
	48	0.595	mmol/l
	49	0.575	mmol/l
	50	0.544	mmol/l
	51	0.580	mmol/l
	52	0.573	mmol/l
	53	0.589	mmol/l
	54	0.570	mmol/l
	55	0.570	mmol/l
	56	0.566	mmol/l
	57	0.617	mmol/l
	58	0.588	mmol/l
	59	0.569	mmol/l
	60	0.569	mmol/l
	61	0.581	mmol/l
	62	0.560	mmol/l
	63	0.567	mmol/l
	64	0.603	mmol/l
	65	0.560	mmol/l
	66	0.558	mmol/l
	67	0.470	mmol/l
	68	0.479	mmol/l
	69	0.489	mmol/l
	70	0.476	mmol/l
	71	0.491	mmol/l
	72	0.476	mmol/l
	73	0.439	mmol/l
	74	0.474	mmol/l
	75	0.440	mmol/l
	76	0.447	mmol/l
	78	0.482	mmol/l
	79	0.403	mmol/l
→	77	0.434	mmol/l
	80	0.391	mmol/l
	81	0.422	mmol/l
	82	0.414	mmol/l
	83	0.380	mmol/l
	84	0.392	mmol/l
	85	0.366	mmol/l
	86	0.532	mmol/l
	87	>0.750	mmol/l

1 2] M.I. ordre glucosa 3 4 5

ANNEX VI: Metabolic Model

The metabolic model used during the experimentation *in silico* is saved in the following Dropbox. I recommend checking the script with the Windows' Notebook or some external program like Geany (the one I used to edit the model).

If you are watching this on a computer screen you can access to the file at this dropbox.

URL: https://www.dropbox.com/s/sl1vq2knlpo34wt/model_with_hb.sbml?dl=0

ANNEX VII: Excel Sheets

In this annex we can find all the tables and graphics done with the excel to arrange and manage all the data from the *in vitro* experimentation.

Counting | COBAS-Glc | COBAS-Lac | COBAS-Glu | Kpc | Kpv

Counting

Cultures					
Growth 0 - 48 h					
		sw480			
Time	Sample	Counting (*10^5)	Dilution	Cell/Well (*10^5)	Volume (pL)
Seeding					
0	1	1,806	1	1,806	0,265
	2	1,588	1	1,588	2,316
	Mean	**1,697**	**1**	**1,697**	**2,316**
	SD			0,154149278	0
	SD/2			0,077074639	
24	1	1,284	2	2,568	1,889
	2	1,554	2	3,108	1,979
	3	1,416	2	2,832	1,461
	Mean	**1,418**	**2**	**2,836**	**1,776333333**
	SD			0,270022221	0,276769459
	SD/2			0,135011111	
24	1bhb	1,469	2	2,938	2,049
	2bhb	1,226	2	2,452	1,938
	3bhb	1,495	2	2,99	1,87
	Mean	**1,396666667**	**2**	**2,793333333**	**1,952333333**
	SD			0,296744559	0,090356701
	SD/2			0,14837228	
48	1	1,099	4	4,396	1,915
	2	1,347	4	5,388	1,984
	3	3,583	4	14,332	1,461
	Mean	**2,009666667**	**4**	**8,038666667**	**1,786666667**
	SD			5,472709506	0,284137877
	SD/2			2,736354753	
48	1bhb	1,207	4	4,828	1,905
	2bhb	2,115	4	8,46	1,469
	3bhb	1,827	4	7,308	1,712
	Mean	**1,716333333**	**4**	**6,865333333**	**1,695333333**
	SD			1,856022988	0,218477306
	SD/2			0,928011494	

Table 7 Proliferation calculation table for sw480.

Diagram 5

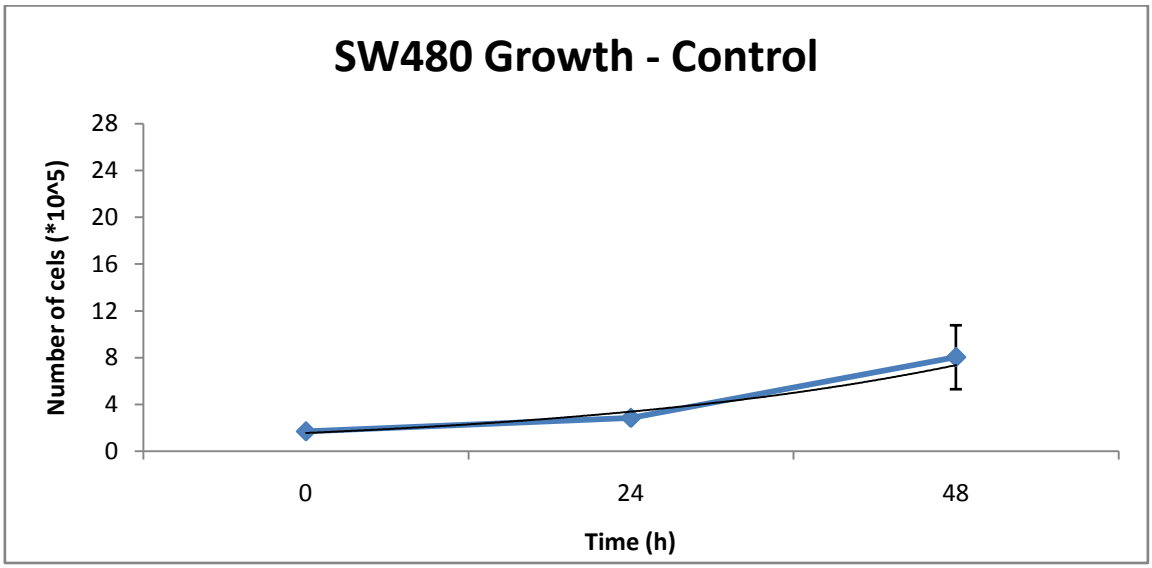


Diagram 6

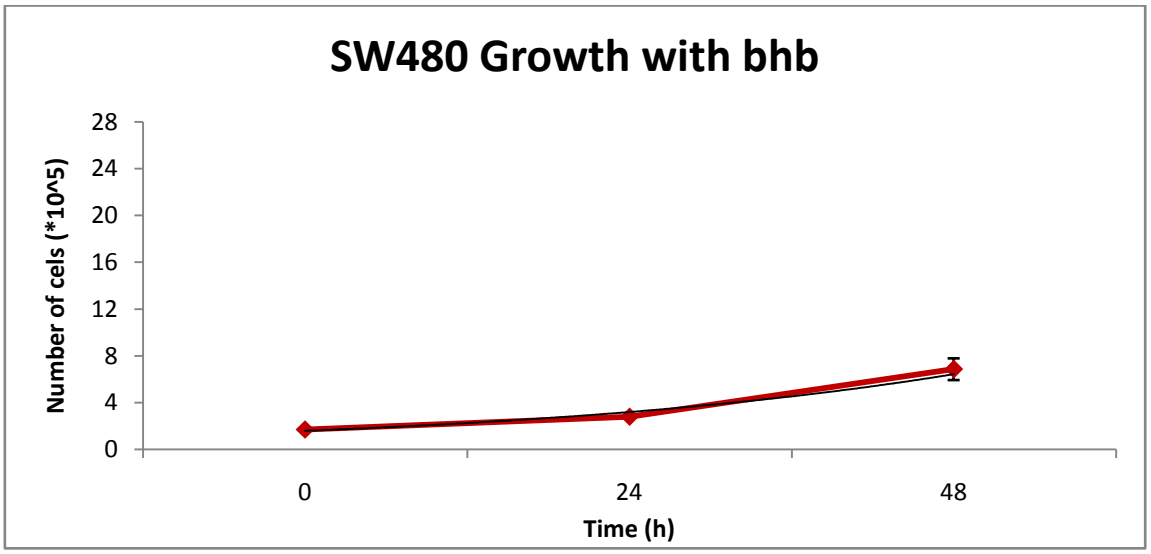
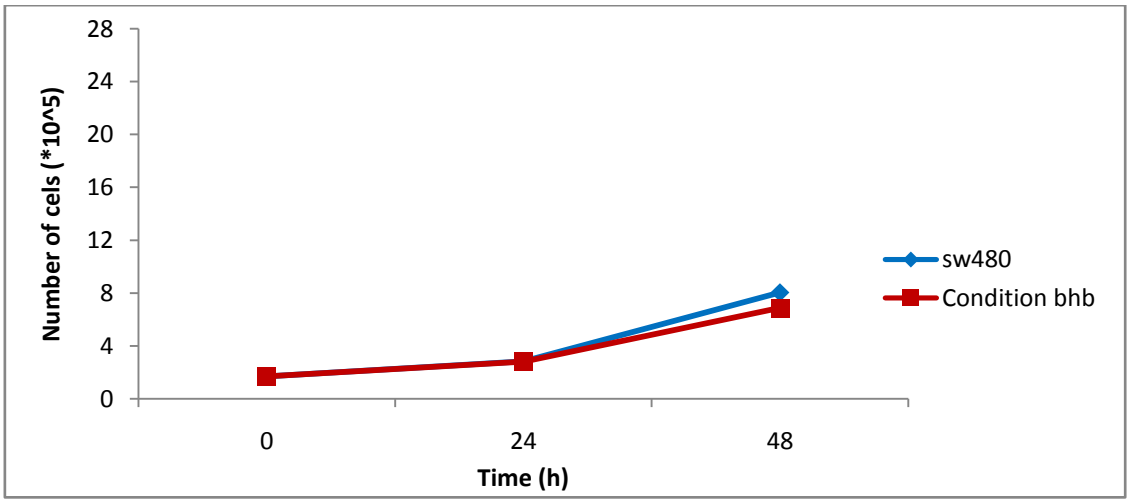


Diagram 7



Time	sw620 Counting (*10^5)	Dilution	Cell/Well (*10^5)	Volume (pL)
Seeding				
0	2,863	1	2,863	1,283
	1,633	1	1,633	1,142
Mean	**2,248**	**1**	**2,248**	**1,2125**
SD			0,869741341	0,099702056
SD/2			0,43487067	
24	3	2	6,044	1,177
	3,256	2	6,512	1,039
	3,043	2	6,086	0,994
Mean	**3,107**	**2**	**6,214**	**1,07**
SD			0,258928562	0,095357223
SD/2			0,129464281	
24	2,745	2	5,49	1,099
	2,6	2	5,2	1,198
	2,766	2	5,532	1,048
Mean	**2,703666667**	**2**	**5,407333333**	**1,115**
SD			0,180779792	0,07626926
SD/2			0,090389896	
48	3,513	8	28,104	1,002
	3,406	8	27,248	0,534
	1,822	8	14,576	1,141
Mean	**2,913666667**	**8**	**23,30933333**	**0,892333333**
SD			7,575388923	0,318013102
SD/2			3,787694461	
48	1,403	8	11,224	1,133
	1,732	8	13,856	0,931
	2,82	8	22,56	0,948
Mean	**1,985**	**8**	**15,88**	**1,004**
SD			5,932845523	0,112040171
SD/2			2,966422762	

Table 8 Proliferation calculation table for sw620.

Diagram 8

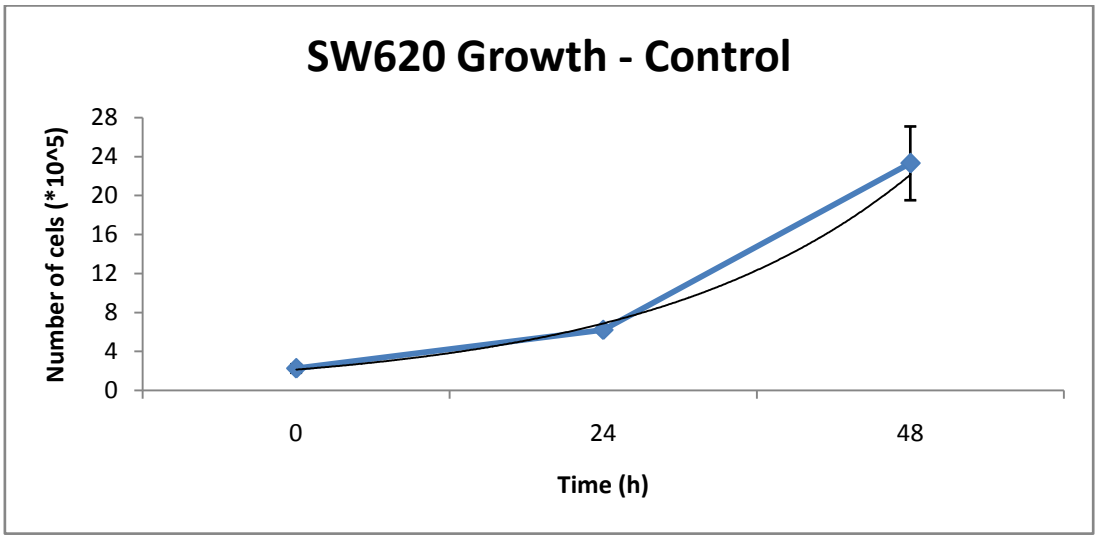


Diagram 9

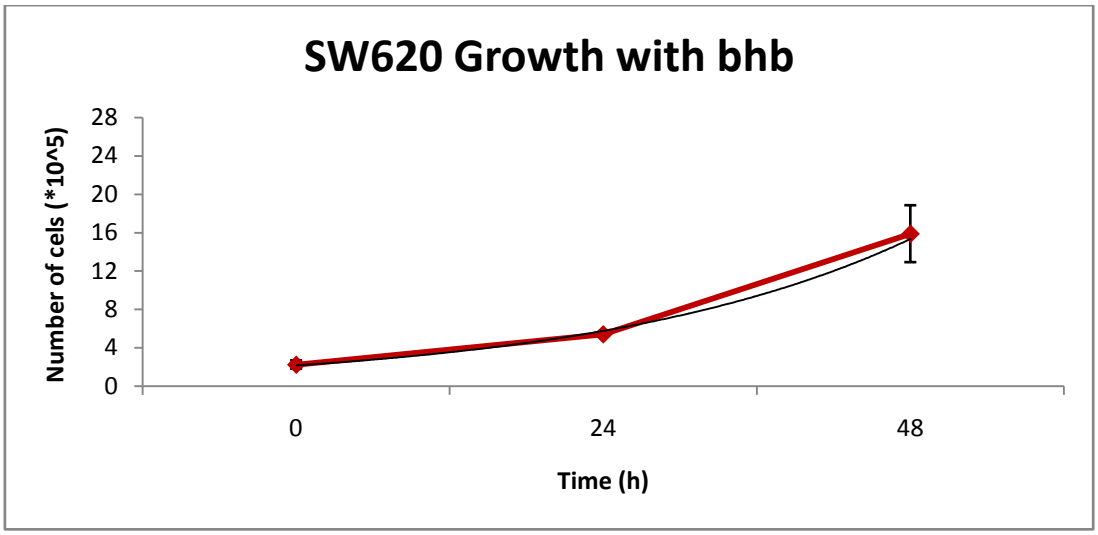
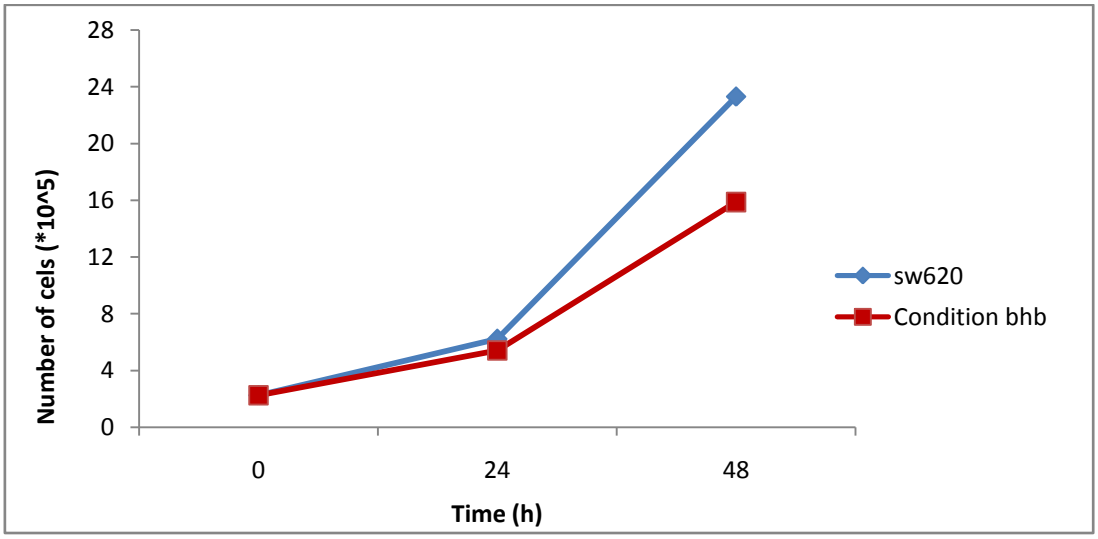


Diagram 10



	sw620-LiM2			
Time	Counting (*10^5)	Dilution	Cell/Well (*10^5)	Volume (pL)
Seeding				
0	3,17	1	3,17	1,477
	3,17	1	3,17	1,56
Mean	**3,17**	**1**	**3,17**	**1,5185**
SD			0	0,058689863
SD/2			0	
24	2,256	2	4,512	1,437
	3,873	2	7,746	1,382
	3,214	2	6,428	1,406
Mean	**3,114333333**	**2**	**6,228666667**	**1,408333333**
SD			1,626188591	0,027574142
SD/2			0,813094295	
24	2,977	2	5,954	1,472
	3,056	2	6,112	1,469
	3,277	2	6,554	0,2
Mean	**3,103333333**	**2**	**6,206666667**	**1,4705**
SD			0,311000536	0,00212132
SD/2			0,155500268	
48	3,479	4	13,916	1,396
	1,838	4	7,352	1,04
	3,932	4	15,728	1,428
Mean	**3,083**	**4**	**12,332**	**1,288**
SD			4,406941797	0,21536945
SD/2			2,203470898	
48	3,172	4	12,688	1,4
	4,678	4	18,712	1,415
	4,056	4	16,224	1,428
Mean	**3,968666667**	**4**	**15,87466667**	**1,414333333**
SD			3,02715532	0,0140119
SD/2			1,51357766	

Table 9 Proliferation calculation table for sw620-LiM2.

Diagram 11

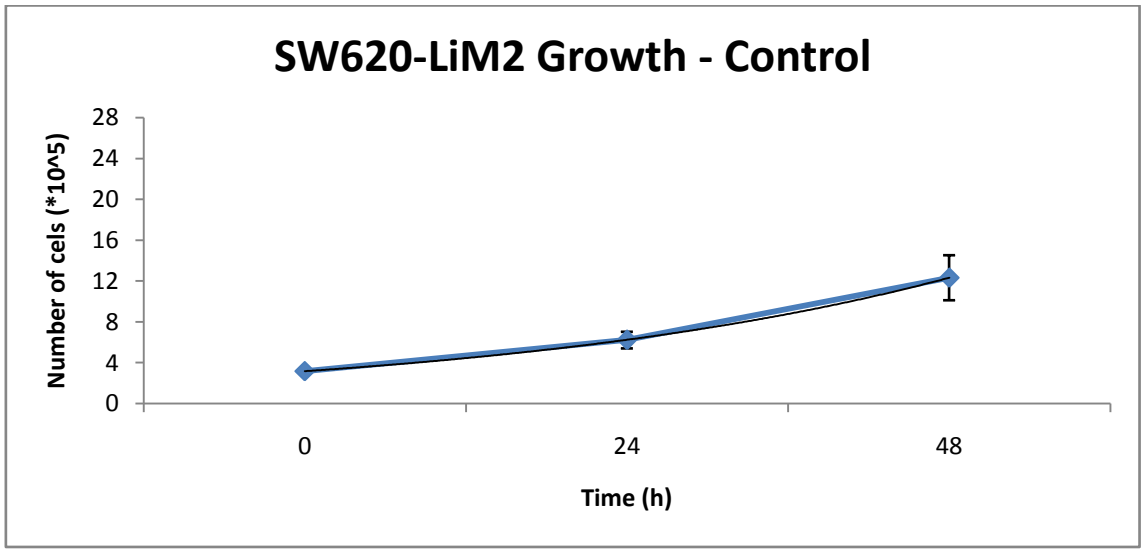


Diagram 12

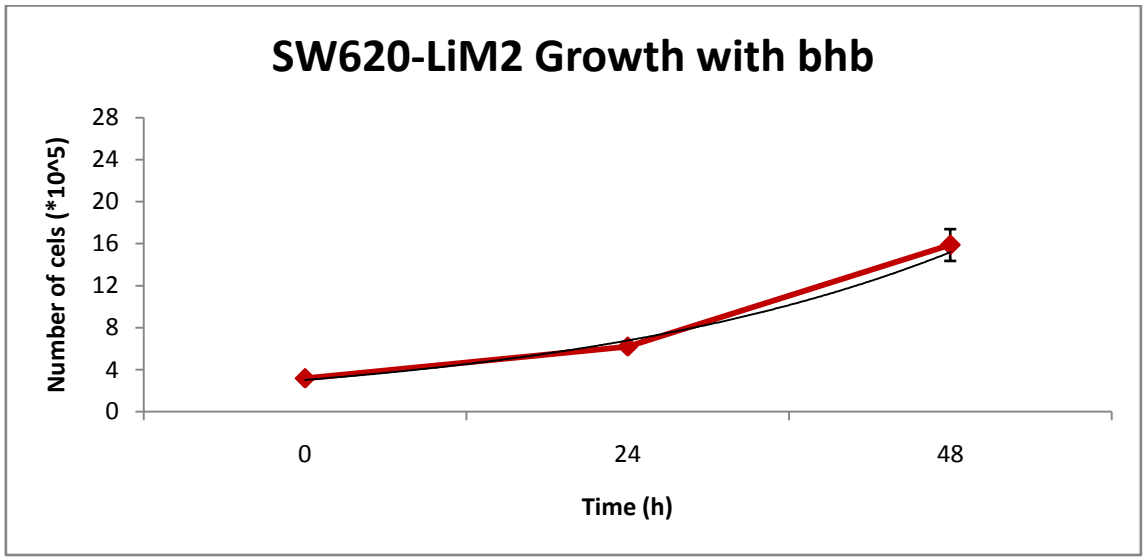
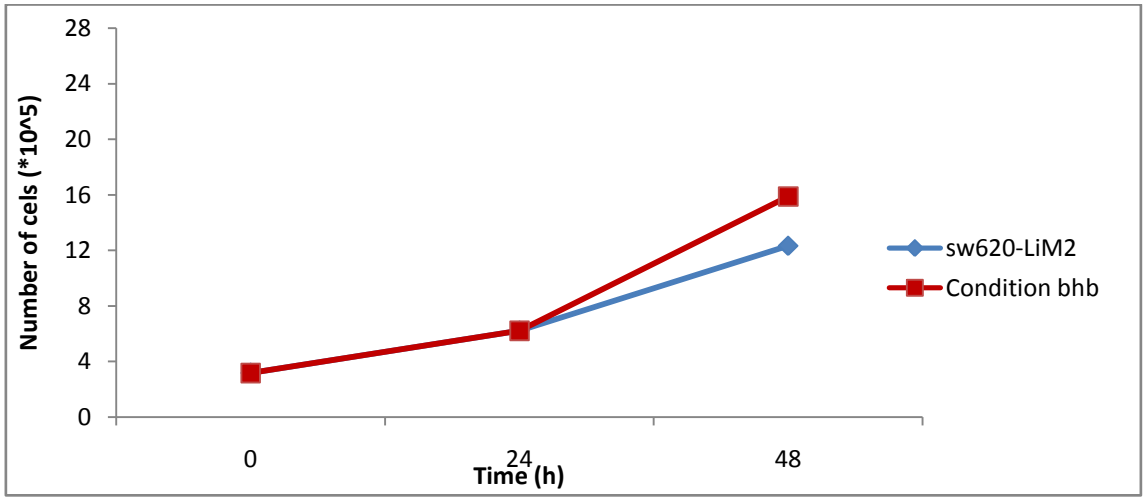


Diagram 13



Proliferation

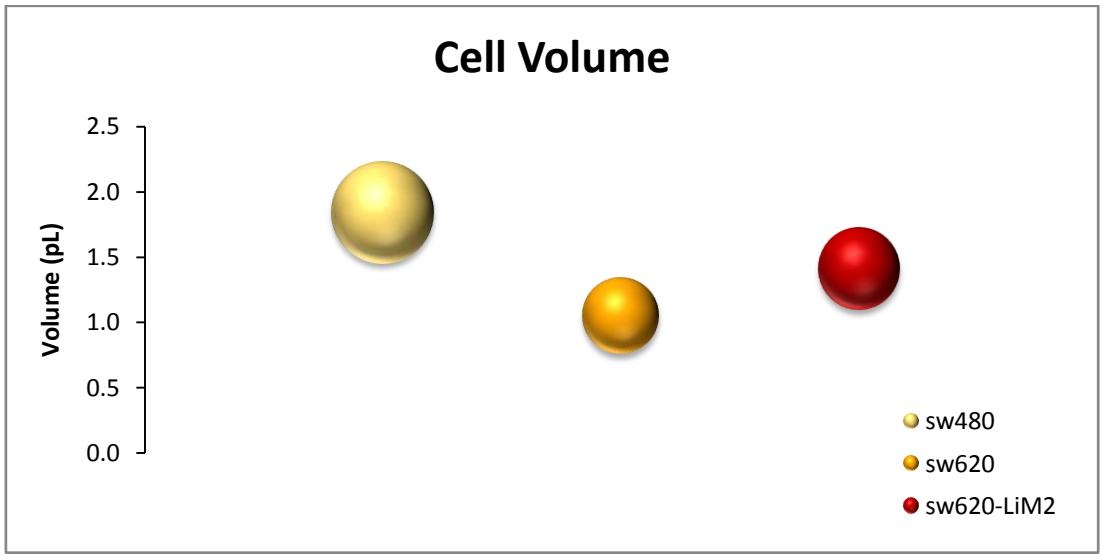
Test T	24h	48h
sw480	0,862822	0,742828
sw620	0,011472	0,252095
sw620-LiM2	0,982741	0,315072

Table 10 Test T for the proliferation at 24 and 48h.

Cell Volume				
		sw480	sw620	sw620-LiM2
Volumes	0h		1,283	1,477
		2,316	1,142	1,56
	24h	1,889	1,177	1,437
		1,979	1,039	1,382
		1,461	0,994	1,406
		2,049	1,099	1,472
		1,938	1,198	1,469
		1,87	1,048	
	48h	1,915	1,002	1,396
		1,984	0,534	1,04
		1,461	1,141	1,428
		1,905	1,133	1,4
		1,469	0,931	1,415
		1,712	0,948	1,428
Average		**1,842154**	**1,047786**	**1,408461538**
SD		0,25402	0,1786	0,120305594

Table 11 Cell volumes Calculations.

Diagram 14



COBAS-Glc

Glc (mM)	18-jul								
			COBAS MEASUREMENTS			CALCULATIONS			
Sample name	COBAS sar	Fisrt	Second	Third	Dilution	Average	Average (line and time)	SD	SD (line and time)
0h Control 1	1	Calc Range	6,81	6,83	2	13,64	**13,64**	0,014142136	
0h bhb	2	Calc Range	6,51	6,5	2	13,01	**13,01**	0,007071068	
480 24h Control 1	3	Calc Range	6,45	6,51	2	12,96	**12,71333333**	0,042426407	0,224796204
480 24h Control 2	4	Calc Range	6,26	6,26	2	12,52		0	
480 24h Control 3	5	Calc Range	6,25	6,41	2	12,66		0,113137085	
480 24h Bhb 1	6	Calc Range	6,35	6,47	2	12,82	**12,79666667**	0,084852814	0,136503968
480 24h Bhb 2	7	Calc Range	6,34	6,31	2	12,65		0,021213203	
480 24h Bhb 3	8	Calc Range	6,44	6,48	2	12,92		0,028284271	
620 24h Control 1	9	Calc Range	6,19	6,18	2	12,37	**12,17666667**	0,007071068	0,172143351
620 24h Control 2	10	Calc Range	6,04	6	2	12,04		0,028284271	
620 24h Control 3	11	Calc Range	6,04	6,08	2	12,12		0,028284271	
620 24h Bhb 1	12	Calc Range	6,2	6,3	2	12,5	**12,4**	0,070710678	0,156204994
620 24h Bhb 2	13	Calc Range	6,05	6,17	2	12,22		0,084852814	
620 24h Bhb 3	14	Calc Range	6,19	6,29	2	12,48		0,070710678	
LiM2 24h Control 1	15	12,21	12,01		1	12,11	**12,06**	0,141421356	0,113578167
LiM2 24h Control 1	16	11,97	11,89		1	11,93		0,056568542	
LiM2 24h Control 1	17	12,12	12,16		1	12,14		0,028284271	
LiM2 24h Bhb 1	18	12,33	12,2		1	12,265	**11,89833333**	0,091923882	0,326279533
LiM2 24h Bhb 2	19	Calc Range	5,89	5,75	2	11,64		0,098994949	
LiM2 24h Bhbl 3	20	Calc Range	5,87	5,92	2	11,79		0,035355339	
480 48h Control 1	21	12,02	11,81		1	11,915	**11,83333333**	0,148492424	0,128679188
480 48h Control 2	22	11,92	11,45		1	11,685		0,332340187	
480 48h Control 3	23	11,98	11,82		1	11,9		0,113137085	
480 48h Bhb 1	24	12,43	11,94		1	12,185	**12,055**	0,346482323	0,153948043
480 48h Bhb 2	25	12,4	11,79		1	12,095		0,431335137	
480 48h Bhb 3	26	12,06	11,71		1	11,885		0,247487373	
620 48h Control 1	27	9,81	9,55		1	9,68	**9,5**	0,183847763	0,209523268
620 48h Control 2	28	9,38	9,16		1	9,27		0,155563492	
620 48h Control 3	29	9,58	9,52		1	9,55		0,042426407	
620 48h Bhb 1	30	9,7	9,78		1	9,74	**9,65**	0,056568542	0,200748599
620 48h Bhb 2	31	9,32	9,52		1	9,42		0,141421356	
620 48h Bhb 3	32	9,74	9,84		1	9,79		0,070710678	
LiM2 48h Control 1	33	8,04	8,15		1	8,095	**8,025**	0,077781746	0,061441029
LiM2 48h Control 1	34	7,92	8,04		1	7,98		0,084852814	
LiM2 48h Control 1	35	7,79	8,21		1	8		0,296984848	
LiM2 48h Bhb 1	36	8,5	8,4		1	8,45	**8,338333333**	0,070710678	0,103722386
LiM2 48h Bhb 2	37	8,22	8,42		1	8,32		0,141421356	
LiM2 48h Bhbl 3	38	8,24	8,25		1	8,245		0,007071068	

Table 12 Glucose concentrations calculations.

Standard line COBAS	Concentrations (mM)
STD-1	**0,5**
STD-11	0,5
STD-12	0,5
STD-2	**2,51**
STD-21	2,52
STD-22	2,5
STD-3	**5,05**
STD-31	5,04
STD-32	5,06
STD-4	**7,67**
STD-41	7,59
STD-42	7,75
STD-5	**9,655**
STD-51	9,59
STD-52	9,72
STD-6	**12,5**
STD-61	12,5
STD-62	12,5

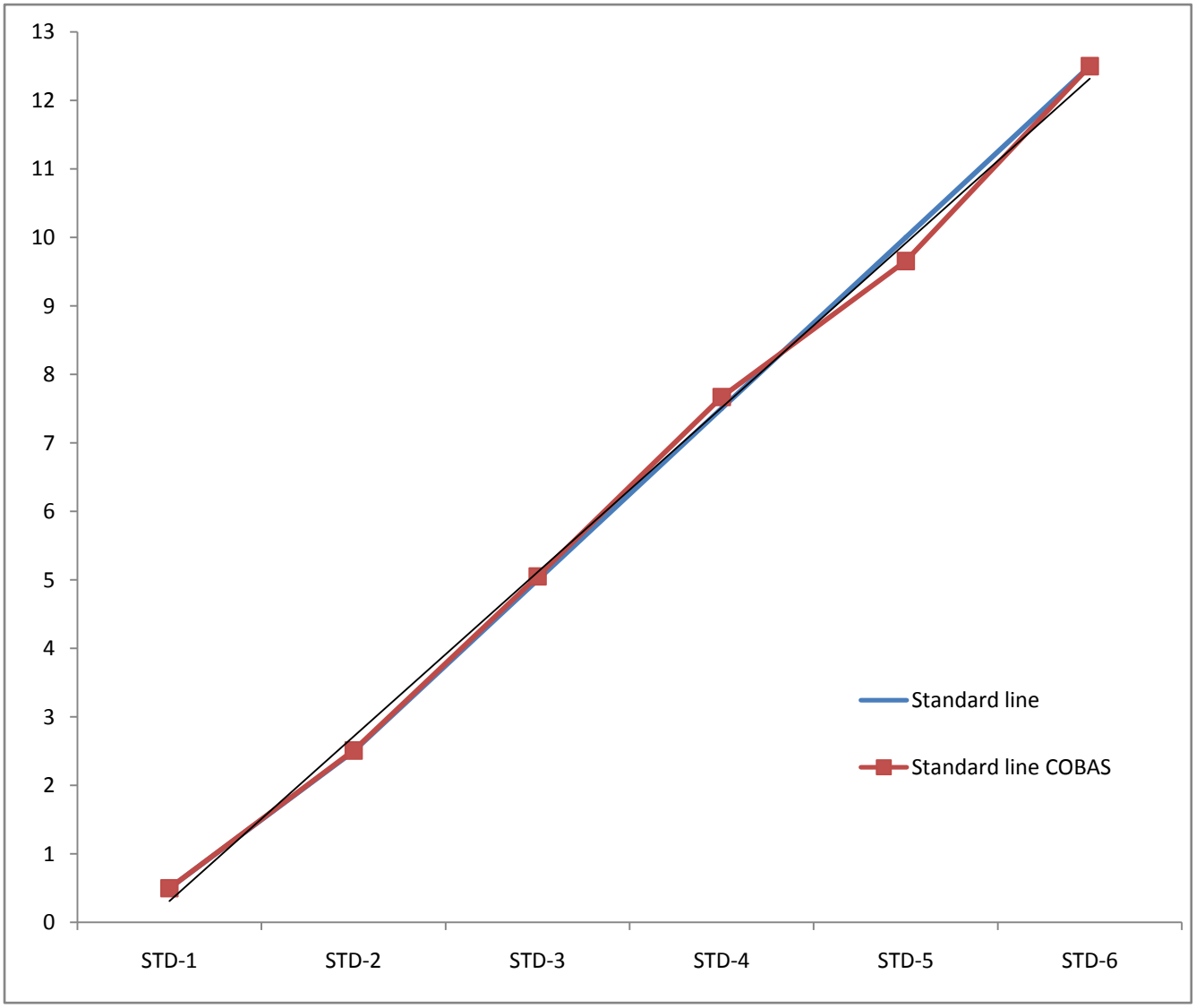
Pendent (A)	0,09131
Ordenada a 0 (R_0)	0,0531

Standard line
0,5
2,5
5
7,5
10
12,5

Pendent (A)	0,0922 ± 0,003
Ordenada a 0 (R_0)	0,087 ± 0,002

Table 13 Glucose standard line measured with COBAS and ideal standard line.

Diagram 15 Glucose Spectrophotometry Standard line.



COBAS-Lac

Lac	19-jul								
(mM)									
		COBAS MESUREMENTS				CALCULATIONS			
Sample name	COBAS sample Name	Fisrt	Second	Third	Dilution	Average	Average (line and time)	SD	SD (line and time)
0h Control 1	1	0,505	0,499		1	0,502	**0,502**	0,004242641	
0h bhb	2	0,472	0,48		1	0,476	**0,476**	0,005656854	
480 24h Control 1	3	0,361	Calc Range		10	3,61	**3,193333333**		0,538709879
480 24h Control 2	4	Calc Range	0,52	0,514	5	2,585		0,004242641	
480 24h Control 3	5	0,337	0,34		10	3,385		0,00212132	
480 24h Bhb 1	6	Calc Range	0,504	0,5	5	2,51	**3,03**	0,002828427	0,45310043
480 24h Bhb 2	7	0,437	0,324		10	3,24			
480 24h Bhb 3	8	0,334	0,464		10	3,34			
620 24h Control 1	9	0,376	0,377		10	3,765	**4,013333333**	0,000707107	0,302338111
620 24h Control 2	10	0,435	0,43		10	4,35			
620 24h Control 3	11	0,388	0,397		10	3,925		0,006363961	
620 24h Bhb 1	12	0,379	0,385		10	3,79	**3,833333333**		0,066583281
620 24h Bhb 2	13	0,393	0,389		10	3,91		0,002828427	
620 24h Bhb 3	14	0,371	0,389		10	3,8		0,012727922	
LiM2 24h Control 1	15	0,516	0,513		10	5,145	**5,021666667**	0,00212132	0,110942928
LiM2 24h Control 1	16	0,474	0,499		10	4,99			
LiM2 24h Control 1	17	0,493	0,601		10	4,93			
LiM2 24h Bhb 1	18	0,52	0,489		10	5,2	**5,373333333**		0,436615773
LiM2 24h Bhb 2	19	0,505	0,538		10	5,05			
LiM2 24h Bhbl 3	20	1,204	0,46	0,587	10	5,87			
480 48h Control 1	21	0,729	0,492		10	6,105	**5,676666667**	0,167584307	0,445935347
480 48h Control 2	22	0,52	0,523		10	5,215		0,00212132	
480 48h Control 3	23	0,588	0,554		10	5,71		0,024041631	
480 48h Bhb 1	24	1,231	0,514		10	5,14	**5,43**		0,347383074
480 48h Bhb 2	25	0,954	0,558	0,509	10	5,335		0,034648232	
480 48h Bhb 3	26	0,535	0,628		10	5,815		0,065760931	
620 48h Control 1	27	0,973	0,905		10	9,39	**9,29**	0,048083261	0,08660254
620 48h Control 2	28	1,107	0,915	0,933	10	9,24		0,012727922	
620 48h Control 3	29	0,945	0,903		10	9,24		0,029698485	
620 48h Bhb 1	30	0,915	0,918		10	9,165	**9,376666667**	0,00212132	0,223289797
620 48h Bhb 2	31	1	0,922		10	9,61		0,055154329	
620 48h Bhb 3	32	0,972	0,899		10	9,355		0,051618795	
LiM2 48h Control 1	33	1,145	1,166		10	11,555	**11,805**		0,22220486
LiM2 48h Control 1	34	1,214	1,196	1,184	10	11,98			
LiM2 48h Control 1	35	1,189	1,187		10	11,88		0,001414214	
LiM2 48h Bhb 1	36	1,076	1,078		10	10,77	**11,34722222**	0,001414214	0,812746259
LiM2 48h Bhb 2	37	1,153	1,046		10	10,995		0,075660426	
LiM2 48h Bhbl 3	38	1,291	1,238	1,154	10	12,276667		0,05939697	

Table 14 Lactate concentrations calculations.

Standard line COBAS	Concentrations (mM)
STD-1	**0,321**
STD-11	0,487
STD-12	0,408
STD-13	0,321
STD-2	**0,382**
STD-21	Calc Range
STD-22	0,382
STD-23	Calc Range
STD-3	**0,932333333**
STD-31	0,923
STD-32	0,937
STD-33	0,937
STD-4	**1,425333333**
STD-41	1,417
STD-42	1,46
STD-43	1,399
STD-5	**1,778666667**
STD-51	1,742
STD-52	1,752
STD-53	1,842
STD-6	**2,21**
STD-61	2,161
STD-62	2,148
STD-63	Calc Range

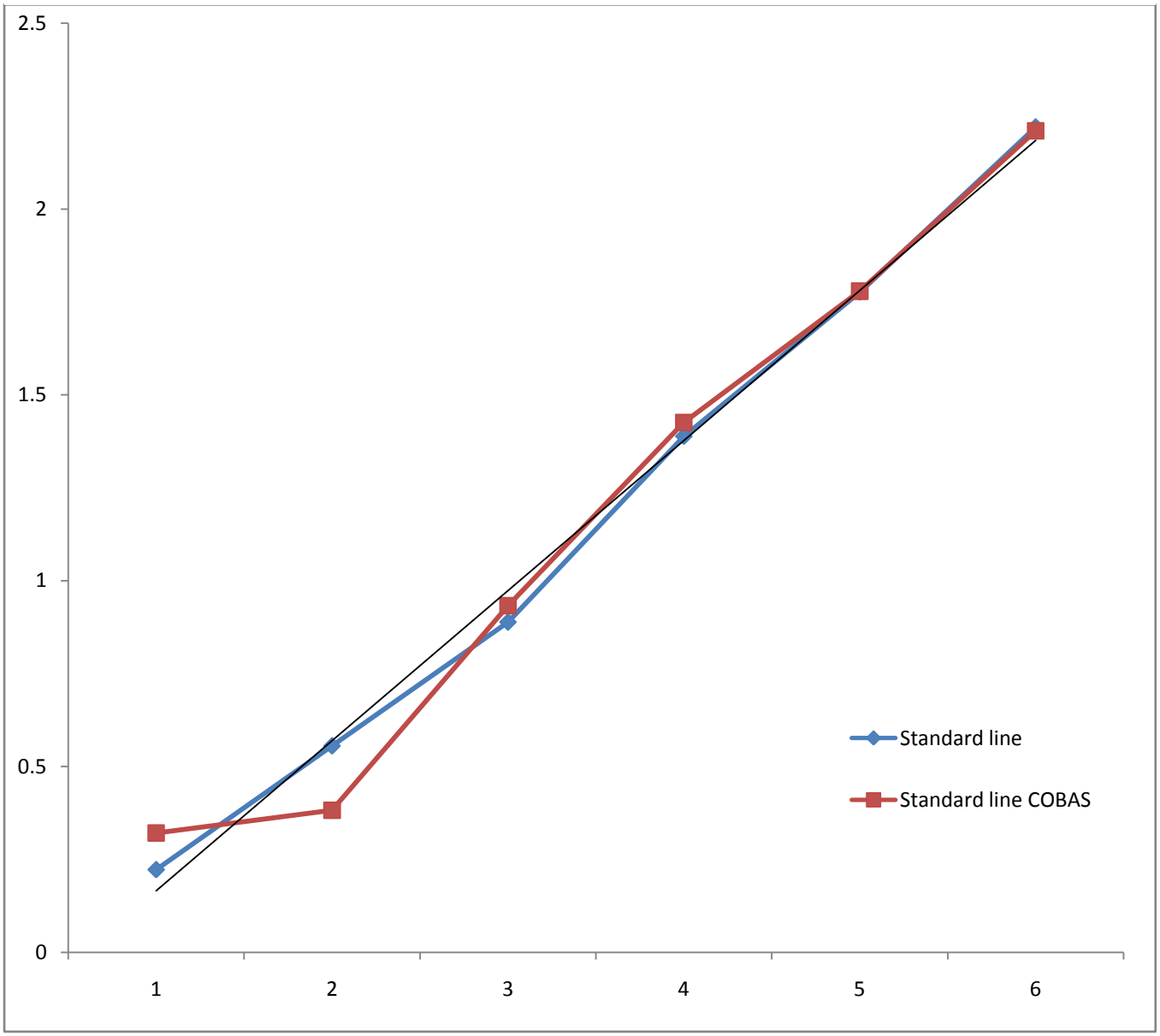
Pendent (A)	0,1776
Ordenada a 0 (R_0)	:-0,2273

Standard line
0,222
0,555
0,888
1,388
1,776
2,22

Pendent (A)	0,0018 ± 0,0021
Ordenada a 0 (R_0)	:-0,02 ± 0,02

Table 15 Lactate Standard line measured and the ideal one.

Diagram 16 Lactate spectrophotometry standard line.



COBAS-Glu

Standard line COBAS	Concentrations (mM)
STD-1	**0,06**
STD-11	0,06
STD-12	0,06
STD-13	0,06
STD-2	**0,175**
STD-21	0,175
STD-22	0,177
STD-23	0,175
STD-3	**0,334**
STD-31	0,298
STD-32	0,314
STD-33	0,334
STD-4	**0,45**
STD-41	0,46
STD-42	0,443
STD-43	0,45
STD-5	**0,603**
STD-51	0,601
STD-52	0,6
STD-53	0,603
STD-6	**0,742**
STD-61	0,744
STD-62	0,75
STD-63	0,742

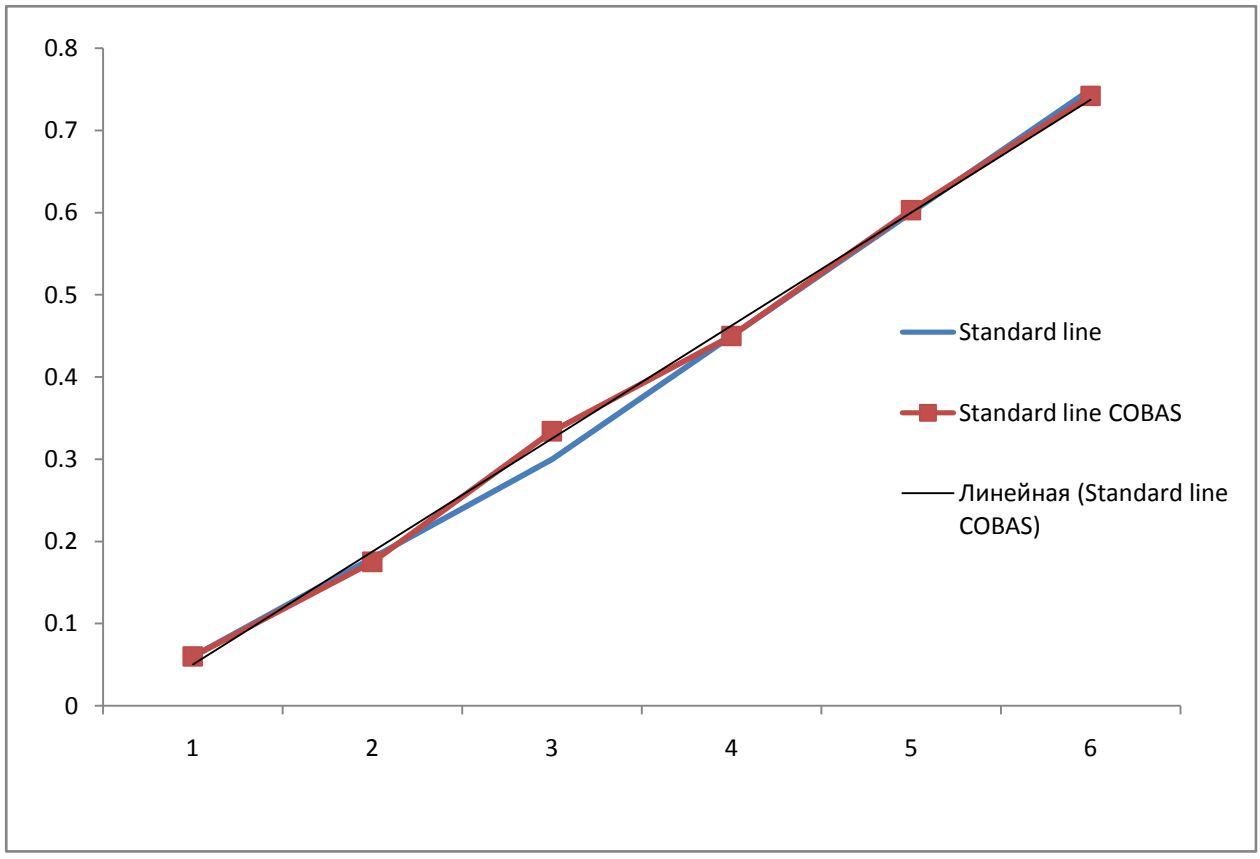
Pendent (A)	0,49792
Ordenada a 0 (R_0)	0,0039

Standard line
0,06
0,18
0,3
0,45
0,6
0,75

Pendent (A)	0,516 ± 0,008
Ordenada a 0 (R_0)	0,0033 ± 0,0005

Table 16 Glutamine and Glutamate Standard line measured and the ideal one.

Diagram 17 Glutamate and Glutamine spectrophotometry standard line.



Gln (mM)	20-jul								
		COBAS MESUREMENTS				CALCULATIONS			
Sample name	COBAS sample Name	Fisrt	Second	third	Dilution	Average	Average (line and time)	SD	SD (line and time)
0h Control 1	47	0,629	0,623		5	3,13	**3,13**	0,004242641	
0h bhb	48	0,595	0,631		5	3,065	**3,065**		
480 24h Control 1	49	0,575	0,623		5	2,995	**2,944166667**	0,033941125	0,081636899
480 24h Control 2	50	0,544	0,596		5	2,85		0,036769553	
480 24h Control 3	51	0,58	0,615		5	2,9875		0,024748737	
480 24h Bhb 1	52	0,573	0,59		5	2,9075	**2,916666667**	0,012020815	0,029825884
480 24h Bhb 2	53	0,589	0,591		5	2,95		0,001414214	
480 24h Bhb 3	54	0,57	0,587		5	2,8925		0,012020815	
620 24h Control 1	55	0,57	0,586		5	2,89	**2,924166667**	0,011313708	0,091184337
620 24h Control 2	56	0,566	0,576		5	2,855		0,007071068	
620 24h Control 3	57	0,617	0,594		5	3,0275		0,016263456	
620 24h Bhb 1	58	0,588	0,604		5	2,98	**2,914166667**	0,011313708	0,059283921
620 24h Bhb 2	59	0,569	0,59		5	2,8975		0,014849242	
620 24h Bhb 3	60	0,569	0,577		5	2,865		0,005656854	
LiM2 24h Control 1	61	0,581	0,584	0,579	5	2,9066667	**2,879722222**	0,002516611	0,033295117
LiM2 24h Control 1	62	0,56	0,577		5	2,8425		0,012020815	
LiM2 24h Control 1	63	0,567	0,589		5	2,89		0,015556349	
LiM2 24h Bhb 1	64	0,603	0,572	0,574	5	2,915	**2,8325**	0,017349352	0,108195425
LiM2 24h Bhb 2	65	0,56	0,524		5	2,71		0,025455844	
LiM2 24h Bhbl 3	66	0,558	0,591		5	2,8725		0,023334524	
480 48h Control 1	67	0,47	0,481		5	2,3775	**2,408333333**	0,007778175	0,028099526
480 48h Control 2	68	0,479	0,487		5	2,415		0,005656854	
480 48h Control 3	69	0,489	0,484		5	2,4325		0,003535534	
480 48h Bhb 1	70	0,476	0,483		5	2,3975	**2,399166667**	0,004949747	0,007637626
480 48h Bhb 2	71	0,491	0,472		5	2,4075		0,013435029	
480 48h Bhb 3	72	0,476	0,481		5	2,3925		0,003535534	
620 48h Control 1	73	0,439	0,438		5	2,1925	**2,2275**	0,000707107	0,040926764
620 48h Control 2	74	0,474	0,435		5	2,2725		0,027577164	
620 48h Control 3	75	0,44	0,447		5	2,2175		0,004949747	
620 48h Bhb 1	76	0,447	0,5		5	2,3675	**2,216666667**	0,037476659	0,134915097
620 48h Bhb 2	77	0,434	0,436		5	2,175		0,001414214	
620 48h Bhb 3	78	0,403	0,44		5	2,1075		0,026162951	
LiM2 48h Control 1	79	0,482	0,406		5	2,22	**2,069166667**	0,053740115	0,134311516
LiM2 48h Control 1	80	0,391	0,394		5	1,9625		0,00212132	
LiM2 48h Control 1	81	0,422	0,388		5	2,025		0,024041631	
LiM2 48h Bhb 1	82	0,414	0,406	0,401	5	2,035	**1,970833333**	0,006557439	0,060432469
LiM2 48h Bhb 2	83	0,38	0,386		5	1,915		0,004242641	
LiM2 48h Bhbl 3	84	0,392	0,393		5	1,9625		0,000707107	
Calibració 1	85	0,366	0,396		5	**1,905**		0,021213203	
Calibració 2	86	0,532	0,535		5	**2,6675**		0,00212132	
Calibració 3	87	0,75	0,75		5	**3,75**		0	

Table 17 Glutamine concentrations calculations.

Gln Glu(glutaminase)- Gl	Average
3,0165	**3,0165**
2,9735	**2,9735**
2,901	**2,850333333**
2,756	
2,894	
2,8125	**2,818**
2,8465	
2,795	
2,7755	**2,803333333**
2,724	
2,9105	
2,8605	**2,796777778**
2,7835	
2,746333333	
2,774666667	**2,758722222**
2,7295	
2,772	
2,7935	**2,714555556**
2,594666667	
2,7555	
2,250166667	**2,280888889**
2,288	
2,3045	
2,2635	**2,263722222**
2,2725	
2,255166667	
1,997	**2,0385**
2,083	
2,0355	
2,1625	**2,021166667**
1,988	
1,913	
2,019	**1,865166667**
1,754	
1,8225	
1,8315	**1,765**
1,72	
1,7435	

Table 18 As in Spectrophotometry we turn Gln into Glu we must subtract the Glu values to the Gln ones to get the [Gln].

Glu (mM)	20-jul								
		COBAS MESUREMENTS				CALCULATIONS			
Sample name	COBAS sample Name	Fisrt	Second	Third	Dilution	Average	Average (line and time)	SD	SD (line and time)
0h Control 1	1	0,13		0,097	1	0,1135	**0,1135**	0,023334524	
0h bhb	2	0,093		0,09	1	0,0915	**0,0915**	0,00212132	
480 24h Control 1	3	0,096		0,092	1	0,094	**0,093833333**	0,002828427	0,000288675
480 24h Control 2	4	0,1		0,088	1	0,094		0,008485281	
480 24h Control 3	5	0,094		0,093	1	0,0935		0,000707107	
480 24h Bhb 1	6	0,096		0,094	1	0,095	**0,098666667**	0,001414214	0,004368447
480 24h Bhb 2	7	0,097		0,11	1	0,1035		0,009192388	
480 24h Bhb 3	8	0,099		0,096	1	0,0975		0,00212132	
620 24h Control 1	39	0,106		0,107	1	0,1145	**0,120833333**	0,000707107	0,008892881
620 24h Control 2	40	0,136		0,101	1	0,131		0,024748737	
620 24h Control 3	41	0,106		0,11	1	0,117		0,002828427	
620 24h Bhb 1	42	0,11		0,11	1	0,1195	**0,117388889**	0	0,002964294
620 24h Bhb 2	43	0,107	0,105	0,143	1	0,114		0,021385353	
620 24h Bhb 3	44	0,113	0,108	0,14	1	0,1186667		0,017214335	
LiM2 24h Control 1	15	0,153		0,111	1	0,132	**0,121**	0,029698485	0,009848858
LiM2 24h Control 1	16	0,114		0,112	1	0,113		0,001414214	
LiM2 24h Control 1	17	0,119		0,117	1	0,118		0,001414214	
LiM2 24h Bhb 1	18	0,125		0,118	1	0,1215	**0,117944444**	0,004949747	0,003189973
LiM2 24h Bhb 2	19 o 45	0,11	0,118	0,118	1	0,1153333		0,004618802	
LiM2 24h Bhbl 3	20 o 46	0,115	0,117	0,119	1	0,117		0,002	
480 48h Control 1	21 o 9	0,129	0,13	0,123	1	0,1273333	**0,127444444**	0,003785939	0,000509175
480 48h Control 2	22 o 10	0,126	0,129	0,126	1	0,127		0,001732051	
480 48h Control 3	23 o 11	0,131	0,125	0,128	1	0,128		0,003	
480 48h Bhb 1	24 o 12	0,132	0,141	0,129	1	0,134	**0,135444444**	0,006244998	0,001710534
480 48h Bhb 2	25 o 13	0,135	0,14	0,13	1	0,135		0,005	
480 48h Bhb 3	26 o 14	0,139	0,138	0,135	1	0,1373333		0,002081666	
620 48h Control 1	27	0,182		0,209	1	0,1955	**0,189**	0,019091883	0,006763875
620 48h Control 2	28	0,195		0,184	1	0,1895		0,007778175	
620 48h Control 3	29	0,181		0,183	1	0,182		0,001414214	
620 48h Bhb 1	30	0,227		0,183	1	0,205	**0,1955**	0,031112698	0,009041571
620 48h Bhb 2	31	0,186		0,188	1	0,187		0,001414214	
620 48h Bhb 3	32	0,193		0,196	1	0,1945		0,00212132	
LiM2 48h Control 1	33	0,2		0,202	1	0,201	**0,204**	0,001414214	0,003968627
LiM2 48h Control 1	34	0,218		0,199	1	0,2085		0,013435029	
LiM2 48h Control 1	35	0,208		0,197	1	0,2025		0,007778175	
LiM2 48h Bhb 1	36	0,209		0,198	1	0,2035	**0,205833333**	0,007778175	0,01216895
LiM2 48h Bhb 2	37	0,194		0,196	1	0,195		0,001414214	
LiM2 48h Bhbl 3	38	0,216		0,222	1	0,219		0,004242641	

Table 19 Glutamate concentrations calculations.

Kpc

Kpc - COBAS 21-jul

		N - Cells	(10^5 cells)			
Cell line	Well	0h	24h	48h	ΔN 24h	ΔN 48h
SW480	Control-1	1,806	2,568	4,396	0,871	2,699
	Control-2	1,588	3,108	5,388	1,411	3,691
	Control-3		2,832	14,332	1,135	12,635
	Mean	**1,697**	**2,836**	**8,038667**	**1,139**	**6,341667**
	Bhb-1		2,938	4,828	1,241	3,131
	Bhb-2		2,452	8,46	0,755	6,763
	Bhb-3		2,99	7,308	1,293	5,611
	Mean		2,7933333	6,8653333	**1,096333**	**5,168333**
SW620	Control-1	2,863	6,044	28,104	3,796	25,856
	Control-2	1,633	6,512	27,248	4,264	25
	Control-3		6,086	14,576	3,838	12,328
	Mean	**2,248**	**6,214**	**23,30933**	**3,966**	**21,06133**
	Bhb-1		5,49	11,224	3,242	8,976
	Bhb-2		5,2	13,856	2,952	11,608
	Bhb-3		5,532	22,56	3,284	20,312
	Mean		**5,407333**	**15,88**	**3,159333**	**13,632**
SW620-LiM2	Control-1	3,17	4,512	13,916	1,342	10,746
	Control-2	3,17	7,746	7,352	4,576	4,182
	Control-3		6,428	15,728	3,258	12,558
	Mean	**3,17**	**6,228667**	**12,332**	**3,058667**	**9,162**
	Bhb-1		5,954	12,688	2,784	9,518
	Bhb-2		6,112	18,712	2,942	15,542
	Bhb-3		6,554	16,224	3,384	13,054
	Mean		**6,206667**	**15,87467**	**3,036667**	**12,70467**

Table 20 Calculations of ΔN= ΔNumber of cells

Volum:	**0,003**	mL							
M - Glc	(mM)		(mmol)		M - Lac	(mM)		(mmol)	
0h	24h	48h	ΔM 24h * V	ΔM 48h * V	0h	24h	48h	ΔM 24h * V	ΔM 48h * V
13,64	12,96	11,915	-0,00204	-0,005175	0,502	3,61	6,105	0,009324	0,016809
	12,52	11,685	-0,00336	-0,005865		2,585	5,215	0,006249	0,014139
	12,66	11,9	-0,00294	-0,00522		3,385	5,71	0,008649	0,015624
	12,71333	**12,79667**	**-0,00278**	**-0,00542**		**3,193333**	**5,676667**	**0,008074**	**0,015524**
13,01	12,82	12,185	-0,00057	-0,002475	0,476	2,51	5,14	0,006102	0,013992
	12,65	12,095	-0,00108	-0,002745		3,24	5,335	0,008292	0,014577
	12,92	11,885	-0,00027	-0,003375		3,34	5,815	0,008592	0,016017
	12,79667	**12,055**	**-0,00064**	**-0,00287**		**3,03**	**5,43**	**0,007662**	**0,014862**
13,64	12,37	12,11	-0,00381	-0,00459	0,502	3,765	9,39	0,009789	0,026664
	12,04	11,93	-0,0048	-0,00513		4,35	9,24	0,011544	0,026214
	12,12	12,14	-0,00456	-0,0045		3,925	9,24	0,010269	0,026214
	12,17667	**12,06**	**-0,00439**	**-0,00474**		**4,013333**	**9,29**	**0,010534**	**0,026364**
13,01	12,5	12,265	-0,00153	-0,002235	0,476	3,79	9,165	0,009942	0,026067
	12,22	11,64	-0,00237	-0,00411		3,91	9,61	0,010302	0,027402
	12,48	11,79	-0,00159	-0,00366		3,8	9,355	0,009972	0,026637
	12,4	**11,89833**	**-0,00183**	**-0,00334**		**3,833333**	**9,376667**	**0,010072**	**0,026702**
13,64	12,11	11,915	-0,00459	-0,005175	0,502	5,145	11,555	0,013929	0,033159
	11,93	11,685	-0,00513	-0,005865		4,99	11,98	0,013464	0,034434
	12,14	11,9	-0,0045	-0,00522		4,93	11,88	0,013284	0,034134
	12,06	**11,83333**	**-0,00474**	**-0,00542**		**5,021667**	**11,805**	**0,013559**	**0,033909**
13,01	12,265	12,185	-0,002235	-0,002475	0,476	5,2	10,77	0,014172	0,030882
	11,64	12,095	-0,00411	-0,002745		5,05	10,995	0,013722	0,031557
	11,79	11,885	-0,00366	-0,003375		5,87	12,276667	0,016182	0,035402
	11,89833	**12,055**	**-0,00334**	**-0,00287**		**5,373333**	**11,34722**	**0,014692**	**0,032614**

Table 21 Calculations ΔMGlc and ΔMLac, ΔM= Metabolite moles

M - Glu	(mM)		(mmol)		M - Gln	(mM)		(mmol)	
0h	24h	48h	ΔM 24h * V	ΔM 48h * V	0h	24h	48h	ΔM 24h * V	ΔM 48h * V
0,1135	0,094	0,1273333	-5,85E-05	0,0000415	3,0165	2,901	2,5946667	-0,000346	-0,001266
	0,094	0,127	-5,85E-05	0,0000405		2,756	2,7555	-0,000781	-0,000783
	0,0935	0,128	-0,00006	0,0000435		2,894	2,2501667	-0,000368	-0,002299
	0,093833	**0,127444**	**-5,9E-05**	**4,18E-05**		**2,850333**	**2,533444**	**-0,0005**	**-0,00145**
0,0915	0,095	0,134	0,0000105	0,0001275	2,9735	2,8125	2,288	-0,000483	-0,002057
	0,1035	0,135	0,000036	0,0001305		2,8465	2,3045	-0,000381	-0,002007
	0,0975	0,1373333	0,000018	0,0001375		2,795	2,2635	-0,000536	-0,00213
	0,098667	**0,135444**	**2,15E-05**	**0,000132**		**2,818**	**2,285333**	**-0,00047**	**-0,00206**
0,1135	0,1145	0,135	3E-06	0,0000645	3,0165	2,7755	2,2725	-0,000723	-0,002232
	0,131	0,1373333	0,0000525	0,0000715		2,724	2,2551667	-0,000878	-0,002284
	0,117	0,1955	0,0000105	0,000246		2,9105	1,997	-0,000318	-0,003059
	0,120833	**0,155944**	**0,000022**	**0,000127**		**2,803333**	**2,174889**	**-0,00064**	**-0,00252**
0,0915	0,1195	0,1895	0,000084	0,000294	2,9735	2,724	2,083	-0,000749	-0,002672
	0,114	0,182	0,0000675	0,0002715		2,9105	2,0355	-0,000189	-0,002814
	0,1186667	0,205	0,0000815	0,0003405		2,8605	2,1625	-0,000339	-0,002433
	0,117389	**0,192167**	**7,77E-05**	**0,000302**		**2,831667**	**2,093667**	**-0,00043**	**-0,00264**
0,1135	0,132	0,187	0,0000555	0,0002205	3,0165	2,7835	1,988	-0,000699	-0,003086
	0,113	0,1945	-1,5E-06	0,000243		2,7463333	1,913	-0,00081	-0,003311
	0,118	0,201	0,0000135	0,0002625		2,7746667	2,019	-0,000725	-0,002993
	0,121	**0,194167**	**2,25E-05**	**0,000242**		**2,768167**	**1,973333**	**-0,00075**	**-0,00313**
0,0915	0,1215	0,2085	0,00009	0,000351	2,9735	2,7295	1,754	-0,000732	-0,003659
	0,1153333	0,2025	0,0000715	0,000333		2,772	1,8225	-0,000605	-0,003453
	0,117	0,2035	0,0000765	0,000336		2,7935	1,8315	-0,00054	-0,003426
	0,117944	**0,204833**	**7,93E-05**	**0,00034**		**2,765**	**1,802667**	**-0,00063**	**-0,00351**

Table 22 Calculations ΔMGlu and ΔMGln, ΔM= Metabolite moles

μ	(h^-1)	KPC - 24		(μmol/millioncells*h)		KPC - 48		(μmol/millioncells*h)	
μ - 24	μ - 48	kpc - Glc	kpc - Lac	kpc - Glu	kpc - Gln	kpc - Glc	kpc - Lac	kpc - Glu	kpc - Gln
0,017261058	0,01982986	-0,498193046	2,277035277	-0,014286418	-0,08462	-0,564665	1,834096	0,004528228	-0,138084
0,025213227	0,02406901	-0,506521533	0,942039601	-0,008818902	-0,117811	-0,467958	1,1281263	0,003231425	-0,062474
0,021338383	0,04445068	-0,550981628	1,620897993	-0,011244523	-0,068873	-0,121669	0,3641665	0,001013905	-0,053585
0,0212709	**0,0294498**	**-0,5185654**	**1,6133243**	**-0,011449948**	**-0,09043**	**-0,38476**	**1,108796**	**0,002924519**	**-0,08471**
0,022869462	0,02178271	-0,094624933	1,012984806	0,001743091	-0,080182	-0,225736	1,276159	0,011628807	-0,187566
0,015335085	0,03346848	-0,294699442	2,262636827	0,009823315	-0,103963	-0,115907	0,6155124	0,00551035	-0,084745
0,023600475	0,03041891	-0,043019737	1,368983624	0,002867982	-0,085322	-0,171768	0,8151715	0,006997944	-0,108405
0,0206017	**0,0285567**	**-0,1441147**	**1,5482018**	**0,004811463**	**-0,08982**	**-0,17114**	**0,902281**	**0,008045701**	**-0,12691**
0,04120938	0,05262231	-0,424975608	1,091886149	0,000334626	-0,080645	-0,084941	0,4934349	0,001193615	-0,041305
0,044316904	0,0519779	-0,476638669	1,146316	0,005213235	-0,087136	-0,098185	0,5017174	0,001368459	-0,043714
0,041497922	0,03894449	-0,50306616	1,132891753	0,001158376	-0,035082	-0,174657	1,0174348	0,009547912	-0,118708
0,0423414	**0,0478482**	**-0,4682268**	**1,123698**	**0,002235413**	**-0,06762**	**-0,11926**	**0,670862**	**0,004036662**	**-0,06791**
0,037203638	0,03350028	-0,17251785	1,121027752	0,009471568	-0,084398	-0,099129	1,1561512	0,0130398	-0,118489
0,034942404	0,03788911	-0,293486148	1,275735992	0,008358783	-0,023405	-0,140958	0,9397911	0,009311484	-0,09651
0,037521187	0,04804453	-0,176990351	1,110030054	0,009072147	-0,037736	-0,071736	0,5220824	0,006673764	-0,047687
0,0365557	**0,0398113**	**-0,2143314**	**1,1689313**	**0,008967499**	**-0,04851**	**-0,10394**	**0,872675**	**0,009675016**	**-0,08756**
0,014708705	0,03081891	-0,927931024	2,815937088	0,011220081	-0,141312	-0,131171	0,840482	0,005589019	-0,078208
0,037226875	0,01752585	-0,304149337	0,798258612	-8,89326E-05	-0,048053	-0,381995	2,3791138	0,015826908	-0,215617
0,029455494	0,03336898	-0,374728702	1,106199129	0,001124186	-0,060415	-0,11322	1,0053791	0,005693544	-0,064906
0,0271304	**0,0272379**	**-0,535603**	**1,5734649**	**0,004085112**	**-0,08326**	**-0,2088**	**1,408325**	**0,00903649**	**-0,11958**
0,02626382	0,02889427	-0,22447207	1,423363839	0,009039144	-0,073518	-0,08659	1,0804299	0,012279997	-0,127995
0,027355103	0,0369882	-0,390618825	1,304153653	0,006795437	-0,057452	-0,058813	0,6761229	0,007134675	-0,073982
0,030264332	0,03401583	-0,302415987	1,337075275	0,00632099	-0,044619	-0,086093	0,9030692	0,00857102	-0,087394
0,0279611	**0,0332994**	**-0,3058356**	**1,3548643**	**0,00738519**	**-0,05853**	**-0,07717**	**0,886541**	**0,009328564**	**-0,09646**

		Test T	α=0,05						
		24h				48h			
	sw480	0,008832327	0,909062047	0,005488757	0,9715602	0,1969607	0,6814131	0,072094677	0,3621506
	sw620	0,005161782	0,464132468	0,012020871	0,4813905	0,6795534	0,4718325	0,164442689	0,5826085
	sw620-LiM2	0,320896217	0,74553907	0,420694489	0,4618046	0,2058367	0,3569378	0,941273386	0,6728438

Table 23 Calculations of the correction factor (μ), the Kpc at 24 and 48h and the Student's T Test with the significance differences in green.

Kpv

Kpv - DMEM							
		Kpc	(µmol/millioncells*h)				
	Medium Concentration ΔM (mM)	Kpc sw480	Kpc sw480 bhb	Kpc sw620	Kpc sw620 bhb	Kpc LiM2	Kpc LiM2 bhb
Ala	0,4	9,287731353	11,05064269	4,543704091	5,840860888	5,945844312	5,242079199
Arg	0,39810428	9,243714008	10,99827038	4,522170114	5,813179296	5,917665172	5,217235413
Cys	0,20127796	4,673539049	5,560627042	2,286368726	2,939091411	2,991918534	2,637787518
His	0,2	4,643865676	5,525321343	2,271852046	2,920430444	2,972922156	2,621039599
Ile	0,8015267	18,61091165	22,14346291	9,104750365	11,70401488	11,91438242	10,5041661
Leu	0,8015267	18,61091165	22,14346291	9,104750365	11,70401488	11,91438242	10,5041661
Lys	0,7978142	18,5247099	22,04089914	9,062579112	11,64980439	11,85919756	10,45551306
Met	0,20134228	4,675032517	5,562403985	2,287099353	2,940030621	2,992874626	2,638630445
Phe	0,4	9,287731353	11,05064269	4,543704091	5,840860888	5,945844312	5,242079199
Pro	0	0	0	0	0	0	0
Ser	0,4	9,287731353	11,05064269	4,543704091	5,840860888	5,945844312	5,242079199
Thr	0,79831934	18,53643891	22,05485444	9,068317128	11,65718052	11,86670627	10,46213302
Trp	0,078431934	1,821136831	2,166808195	0,890928749	1,145275039	1,165860172	1,027866024
Tyr	0,39846742	9,252145875	11,0083027	4,526295116	5,818481922	5,923063107	5,221994434
Val	0,8034188	18,65484495	22,19573522	9,126243221	11,73164361	11,94250775	10,52896245
Glygn Lowwe	0	0	0	0	0	0	0
Glygn Upper	0	0	0	0	0	0	0
Bhb	5		138,1330336		73,0107611		65,52598998
Glc							
Lac							
Glu							
Gln							

Table 24 Kpc of the medium metabolites.

Kpv	(µmol/millioncells*h)				
Kpv sw480	Kpv sw480 bhb	Kpv sw620	Kpv sw620 bhb	Kpv LiM2	Kpv LiM2 bhb
5,04178E-06	5,99876E-06	4,33648E-06	5,57448E-06	4,2215E-06	3,72185E-06
5,01788E-06	5,97033E-06	4,31593E-06	5,54806E-06	4,2015E-06	3,70421E-06
2,537E-06	3,01855E-06	2,1821E-06	2,80505E-06	2,1242E-06	1,87281E-06
2,52089E-06	2,99938E-06	2,16824E-06	2,78724E-06	2,1108E-06	1,86092E-06
1,01028E-05	1,20204E-05	8,68952E-06	1,11702E-05	8,4591E-06	7,4579E-06
1,01028E-05	1,20204E-05	8,68952E-06	1,11702E-05	8,4591E-06	7,4579E-06
1,0056E-05	1,19647E-05	8,64927E-06	1,11185E-05	8,42E-06	7,42336E-06
2,53781E-06	3,01951E-06	2,18279E-06	2,80595E-06	2,1249E-06	1,87341E-06
5,04178E-06	5,99876E-06	4,33648E-06	5,57448E-06	4,2215E-06	3,72185E-06
0	0	0	0	0	0
5,04178E-06	5,99876E-06	4,33648E-06	5,57448E-06	4,2215E-06	3,72185E-06
1,00624E-05	1,19723E-05	8,65474E-06	1,11255E-05	8,4253E-06	7,42806E-06
9,88591E-07	1,17624E-06	8,50297E-07	1,09304E-06	8,2775E-07	7,29779E-07
5,02246E-06	5,97578E-06	4,31987E-06	5,55312E-06	4,2053E-06	3,70759E-06
1,01266E-05	1,20488E-05	8,71003E-06	1,11966E-05	8,4791E-06	7,47551E-06
0	0	0	0	0	0
0	0	0	0	0	0
0	7,49845E-05	0	6,9681E-05	0	4,65231E-05
-2,08866E-07	-9,29004E-08	-1,13822E-07	-9,92007E-08	-1,4824E-07	-5,47868E-08
6,01902E-07	4,89797E-07	6,40267E-07	8,32875E-07	9,999E-07	6,29439E-07
1,58755E-09	4,36755E-09	3,85256E-09	9,23377E-09	6,4159E-09	6,62323E-09
-4,59866E-08	-6,88896E-08	-6,4812E-08	-8,35686E-08	-8,4899E-08	-6,8484E-08

Volume			
	sw480	sw620	sw620-LiM2
Average	1,842153846	1,047785714	1,408461538
V*millioncells	1842153,846	1047785,714	1408461,538

Table 25 Kpv calculation of all the metabolites for each time and condition.

ANNEX VIII: R Studio Script

R Studio is a computer program more “userfriendly” than its base code R. It is mostly used for statistical analysis.

Along this annex you can check the appearance and the results I got from it.

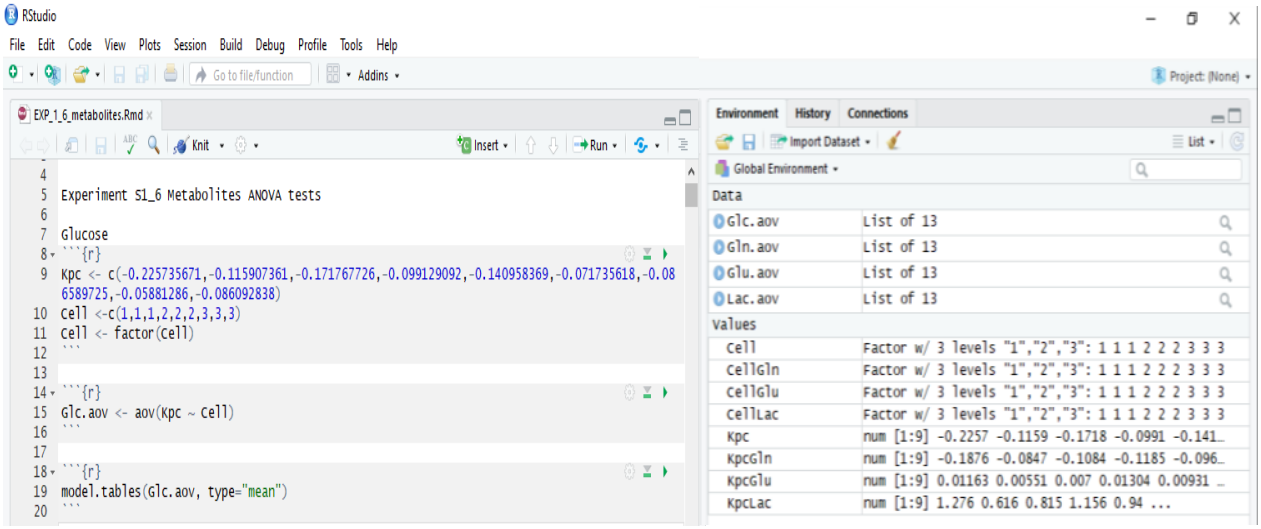


Illustration 57 This are two pictures that show the appearance of a couple of chunks (right) and the data and values stored by the program to ease future calculations that require them (left).

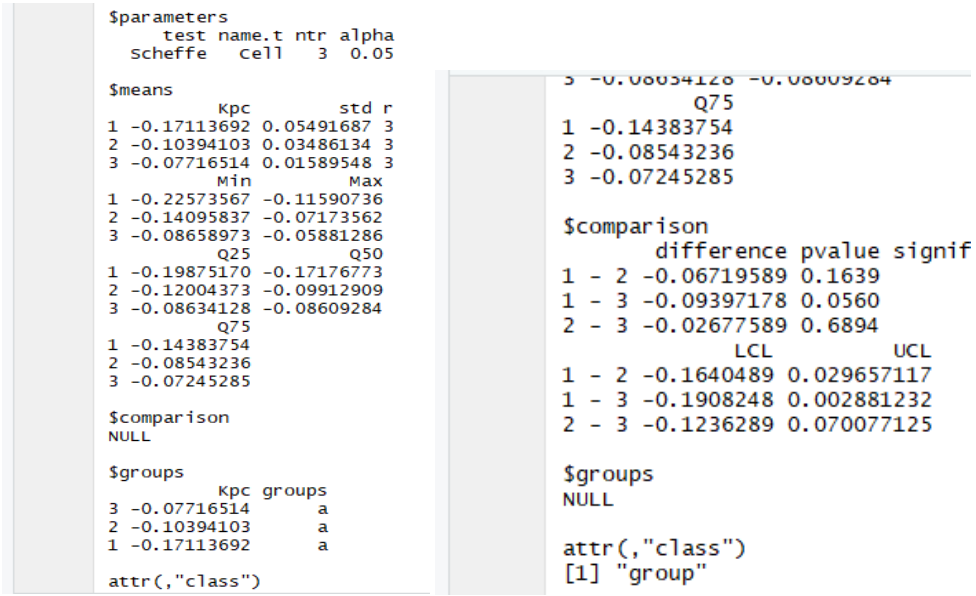


Illustration 58 This two pictures show how the output of most tests: NULL, 1 differentiated group.

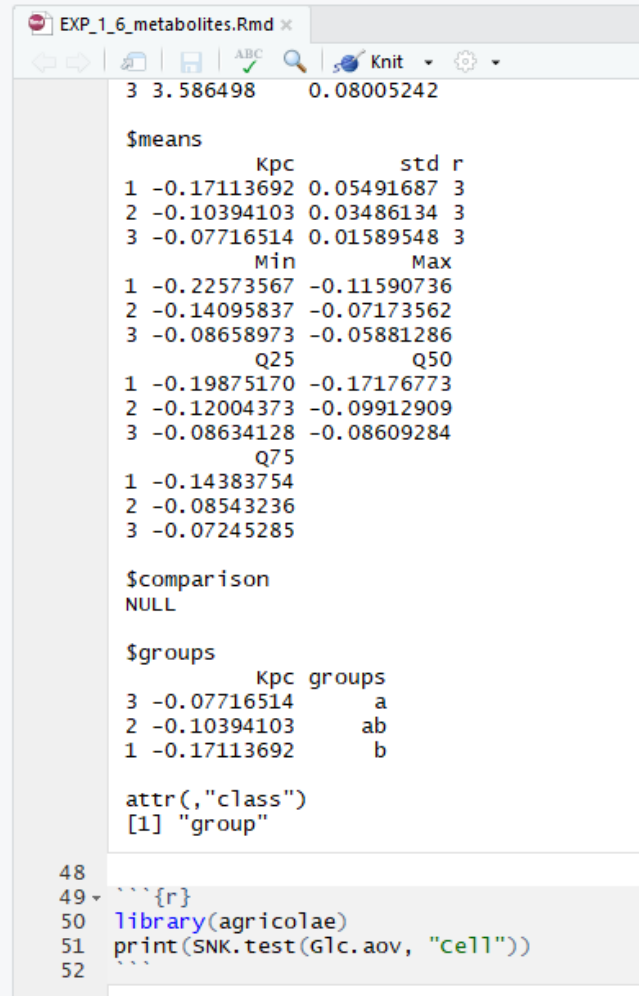
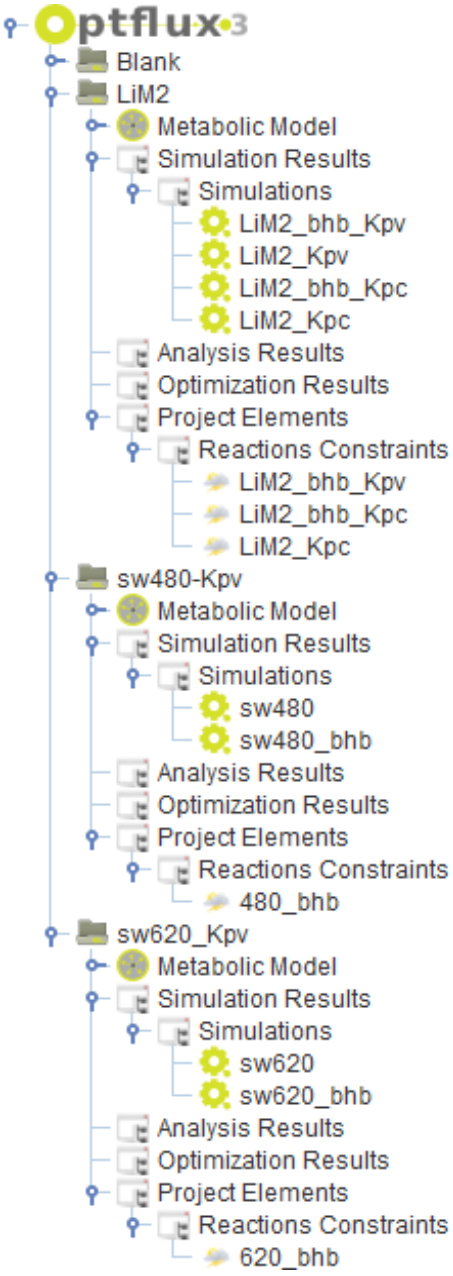


Illustration 59 The only Test that found a differentiated result (3 groups; a, ab and b) and it was between the consumption of Glucose among bhb cell lines.

ANNEX IX: Optflux simulations

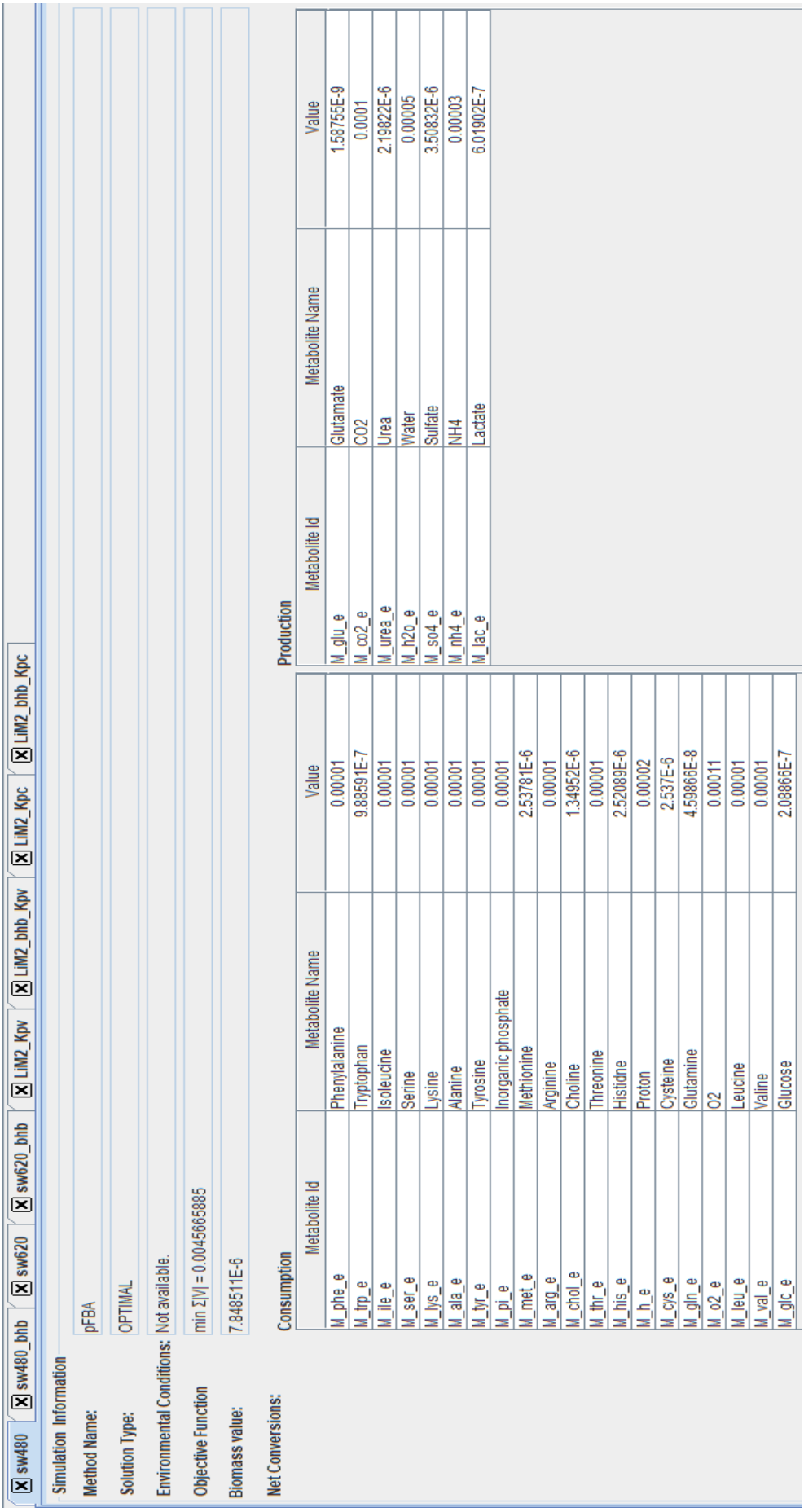


Optflux is free software that you can download from here: http://www.optflux.org/

In this annex, you can check all the simulations ( ) in the following order:

1. SW480
2. SW480 with bhb
3. SW620 bhb
4. SW620
5. LiM2 Kpv
6. LiM2 bhb Kpv
7. LiM2 Kpc
8. LiM2 bhb Kpc

1.



sw480 | sw480_bhb | sw620 | sw620_bhb | LiM2_Kpv | LiM2_bhb_Kpv | LiM2_Kpc | LiM2_bhb_Kpc

Simulation Information

Method Name:	pFBA
Solution Type:	OPTIMAL
Environmental Conditions:	Not available.
Objective Function	min Σ|v| = 0.0045665885
Biomass value:	7.848511E-6

Net Conversions:

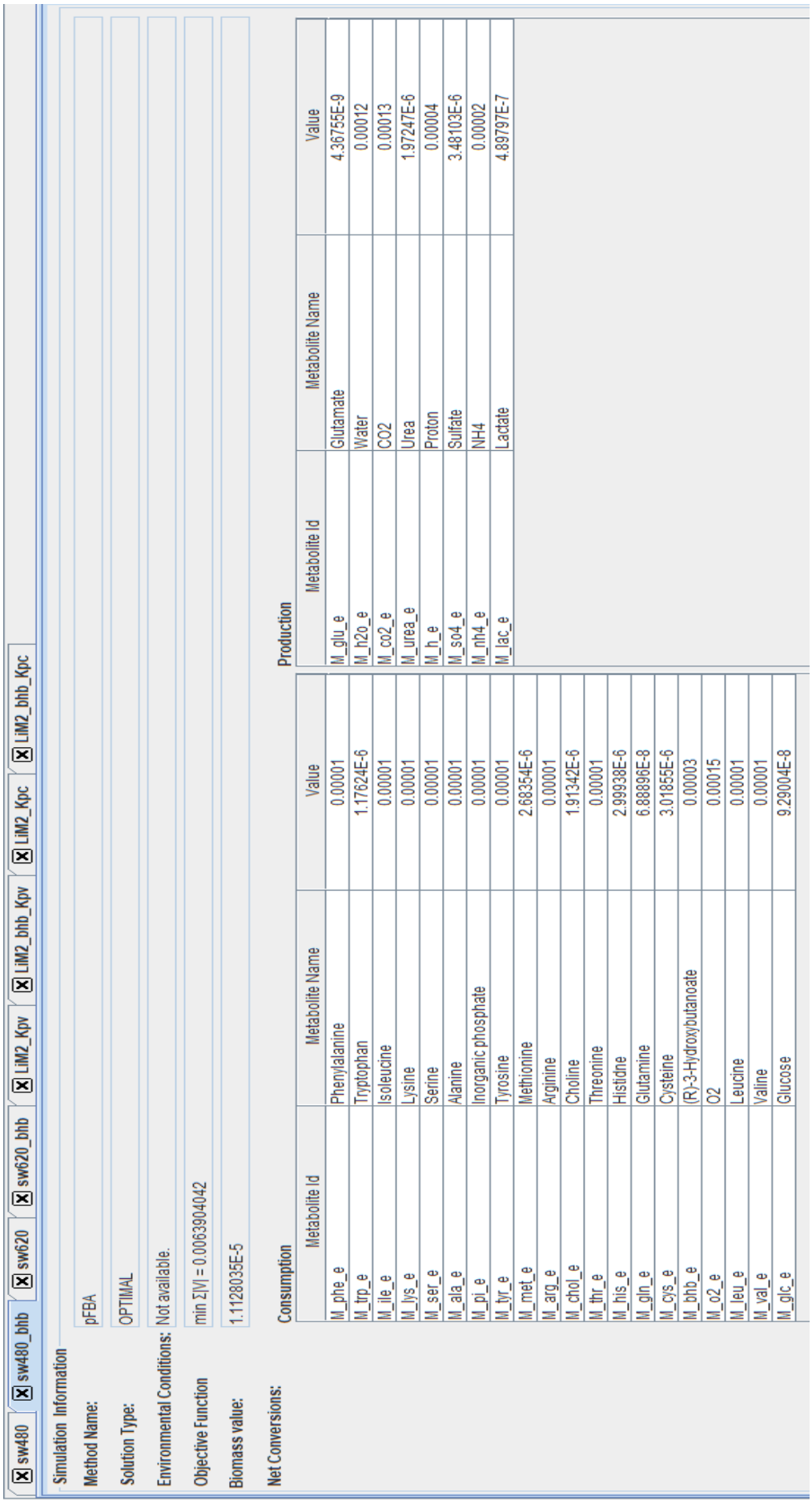
Consumption

Metabolite Id	Metabolite Name	Value
M_phe_e	Phenylalanine	0.00001
M_trp_e	Tryptophan	9.88591E-7
M_ile_e	Isoleucine	0.00001
M_ser_e	Serine	0.00001
M_lys_e	Lysine	0.00001
M_ala_e	Alanine	0.00001
M_tyr_e	Tyrosine	0.00001
M_pi_e	Inorganic phosphate	0.00001
M_met_e	Methionine	2.53781E-6
M_arg_e	Arginine	0.00001
M_chol_e	Choline	1.34952E-6
M_thr_e	Threonine	0.00001
M_his_e	Histidine	2.52089E-6
M_h_e	Proton	0.00002
M_cys_e	Cysteine	2.537E-6
M_gln_e	Glutamine	4.59866E-8
M_o2_e	O2	0.00011
M_leu_e	Leucine	0.00001
M_val_e	Valine	0.00001
M_glc_e	Glucose	2.08866E-7

Production

Metabolite Id	Metabolite Name	Value
M_glu_e	Glutamate	1.58755E-9
M_co2_e	CO2	0.0001
M_urea_e	Urea	2.19822E-6
M_h2o_e	Water	0.00005
M_so4_e	Sulfate	3.50832E-6
M_nh4_e	NH4	0.00003
M_lac_e	Lactate	6.01902E-7

2.



sw480 | sw480_bhb | sw620 | sw620_bhb | LiM2_Kpv | LiM2_bhb_Kpv | LiM2_Kpc | LiM2_bhb_Kpc

Simulation Information

Method Name: pFBA

Solution Type: OPTIMAL

Environmental Conditions: Not available.

Objective Function: min Σ|v| = 0.0063904042

Biomass value: 1.1128035E-5

Net Conversions:

Consumption

Metabolite Id	Metabolite Name	Value
M_phe_e	Phenylalanine	0.00001
M_trp_e	Tryptophan	1.17624E-6
M_ile_e	Isoleucine	0.00001
M_lys_e	Lysine	0.00001
M_ser_e	Serine	0.00001
M_ala_e	Alanine	0.00001
M_pi_e	Inorganic phosphate	0.00001
M_tyr_e	Tyrosine	0.00001
M_met_e	Methionine	2.68354E-6
M_arg_e	Arginine	0.00001
M_chol_e	Choline	1.91342E-6
M_thr_e	Threonine	0.00001
M_his_e	Histidne	2.99938E-6
M_gln_e	Glutamine	6.88896E-8
M_cys_e	Cysteine	3.01855E-6
M_bhb_e	(R)-3-Hydroxybutanoate	0.00003
M_o2_e	O2	0.00015
M_leu_e	Leucine	0.00001
M_val_e	Valine	0.00001
M_glc_e	Glucose	9.29004E-8

Production

Metabolite Id	Metabolite Name	Value
M_glu_e	Glutamate	4.36755E-9
M_h2o_e	Water	0.00012
M_co2_e	CO2	0.00013
M_urea_e	Urea	1.97247E-6
M_h_e	Proton	0.00004
M_so4_e	Sulfate	3.48103E-6
M_nh4_e	NH4	0.00002
M_lac_e	Lactate	4.89797E-7

3.

sw480 | sw480_bhb | sw620 | sw620_bhb | LiM2_Kpv | LiM2_bhb_Kpv | LiM2_Kpc | LiM2_bhb_Kpc

Simulation Information

Method Name:	pFBA
Solution Type:	OPTIMAL
Environmental Conditions:	620_bhb
Objective Function	min Σ|v| = 0.0059081218
Biomass value:	1.0291501E-5

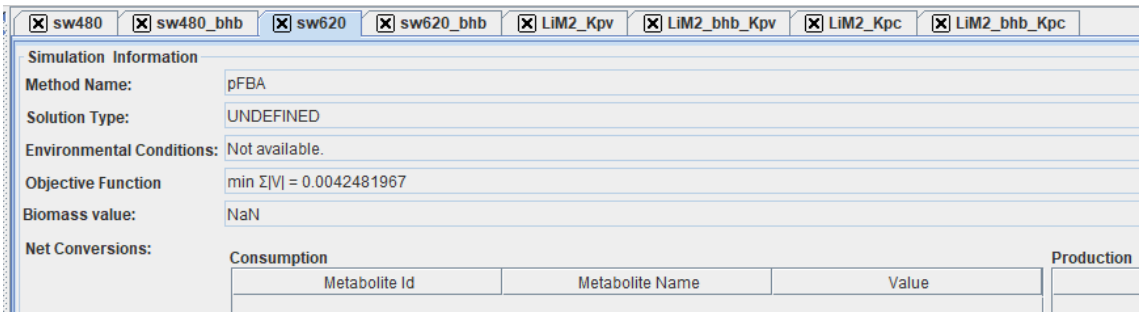
Consumption

Metabolite Id	Metabolite Name	Value
M_phe_e	Phenylalanine	0.00001
M_trp_e	Tryptophan	1.09304E-6
M_ile_e	Isoleucine	0.00001
M_lys_e	Lysine	0.00001
M_ser_e	Serine	0.00001
M_ala_e	Alanine	0.00001
M_pi_e	Inorganic phosphate	0.00001
M_tyr_e	Tyrosine	0.00001
M_met_e	Methionine	2.47668E-6
M_arg_e	Arginine	0.00001
M_chol_e	Choline	1.76958E-6
M_thr_e	Threonine	0.00001
M_his_e	Histidne	2.78724E-6
M_gln_e	Glutamine	8.35686E-8
M_cys_e	Cysteine	2.80505E-6
M_bhb_e	(R)-3-Hydroxybutanoate	0.00003
M_o2_e	O2	0.00014
M_leu_e	Leucine	0.00001
M_val_e	Valine	0.00001
M_glc_e	Glucose	9.92007E-8

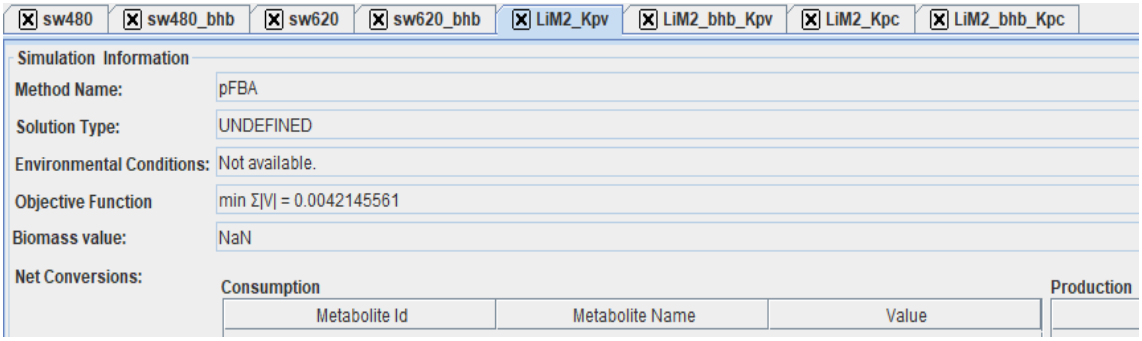
Production

Metabolite Id	Metabolite Name	Value
M_glu_e	Glutamate	9.23377E-9
M_h2o_e	Water	0.00011
M_co2_e	CO2	0.00012
M_urea_e	Urea	1.85074E-6
M_h_e	Proton	0.00003
M_so4_e	Sulfate	3.22764E-6
M_nh4_e	NH4	0.00002
M_lac_e	Lactate	8.32875E-7

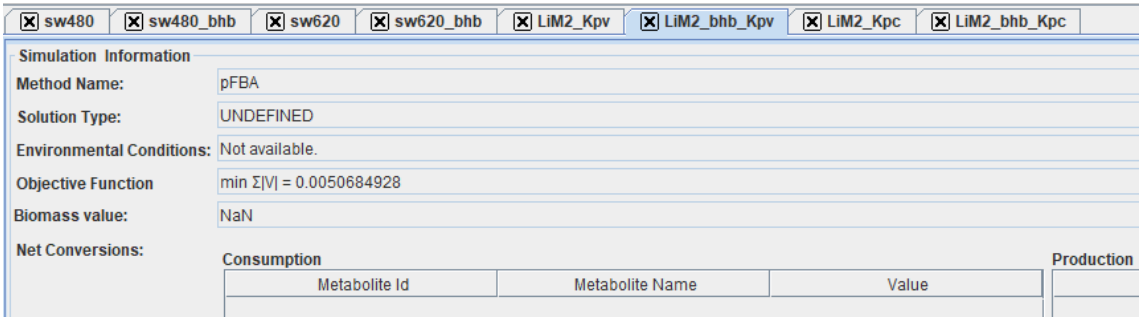
4.


sw480
sw480_bhb
sw620
sw620_bhb
LiM2_Kpv
LiM2_bhb_Kpv
LiM2_Kpc
LiM2_bhb_Kpc
Simulation Information
Method Name: pFBA
Solution Type: UNDEFINED
Environmental Conditions: Not available.
Objective Function min Σ|V| = 0.0042481967
Biomass value: NaN
Net Conversions:
Consumption
Production
Metabolite Id
Metabolite Name
Value

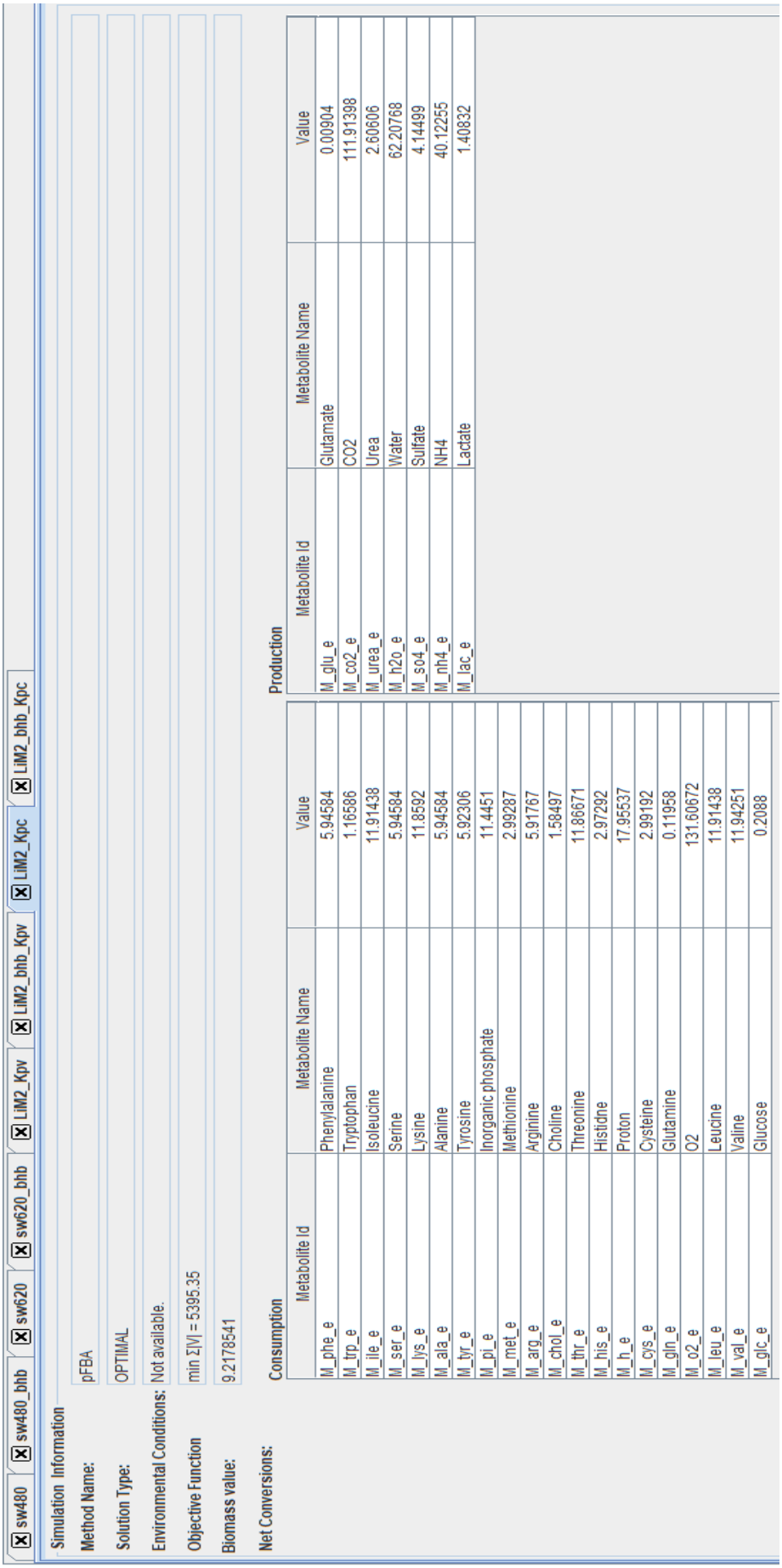
5.


sw480
sw480_bhb
sw620
sw620_bhb
LiM2_Kpv
LiM2_bhb_Kpv
LiM2_Kpc
LiM2_bhb_Kpc
Simulation Information
Method Name: pFBA
Solution Type: UNDEFINED
Environmental Conditions: Not available.
Objective Function min Σ|V| = 0.0042145561
Biomass value: NaN
Net Conversions:
Consumption
Production
Metabolite Id
Metabolite Name
Value

6.


sw480
sw480_bhb
sw620
sw620_bhb
LiM2_Kpv
LiM2_bhb_Kpv
LiM2_Kpc
LiM2_bhb_Kpc
Simulation Information
Method Name: pFBA
Solution Type: UNDEFINED
Environmental Conditions: Not available.
Objective Function min Σ|V| = 0.0050684928
Biomass value: NaN
Net Conversions:
Consumption
Production
Metabolite Id
Metabolite Name
Value

7.



sw480 | sw480_bhb | sw620 | sw620_bhb | LiM2_Kpv | LiM2_bhb_Kpv | LiM2_Kpc | LiM2_bhb_Kpc

Simulation Information

Method Name:	pFBA
Solution Type:	OPTIMAL
Environmental Conditions:	Not available.
Objective Function	min Σ|v| = 5395.35
Biomass value:	9.2178541

Net Conversions:

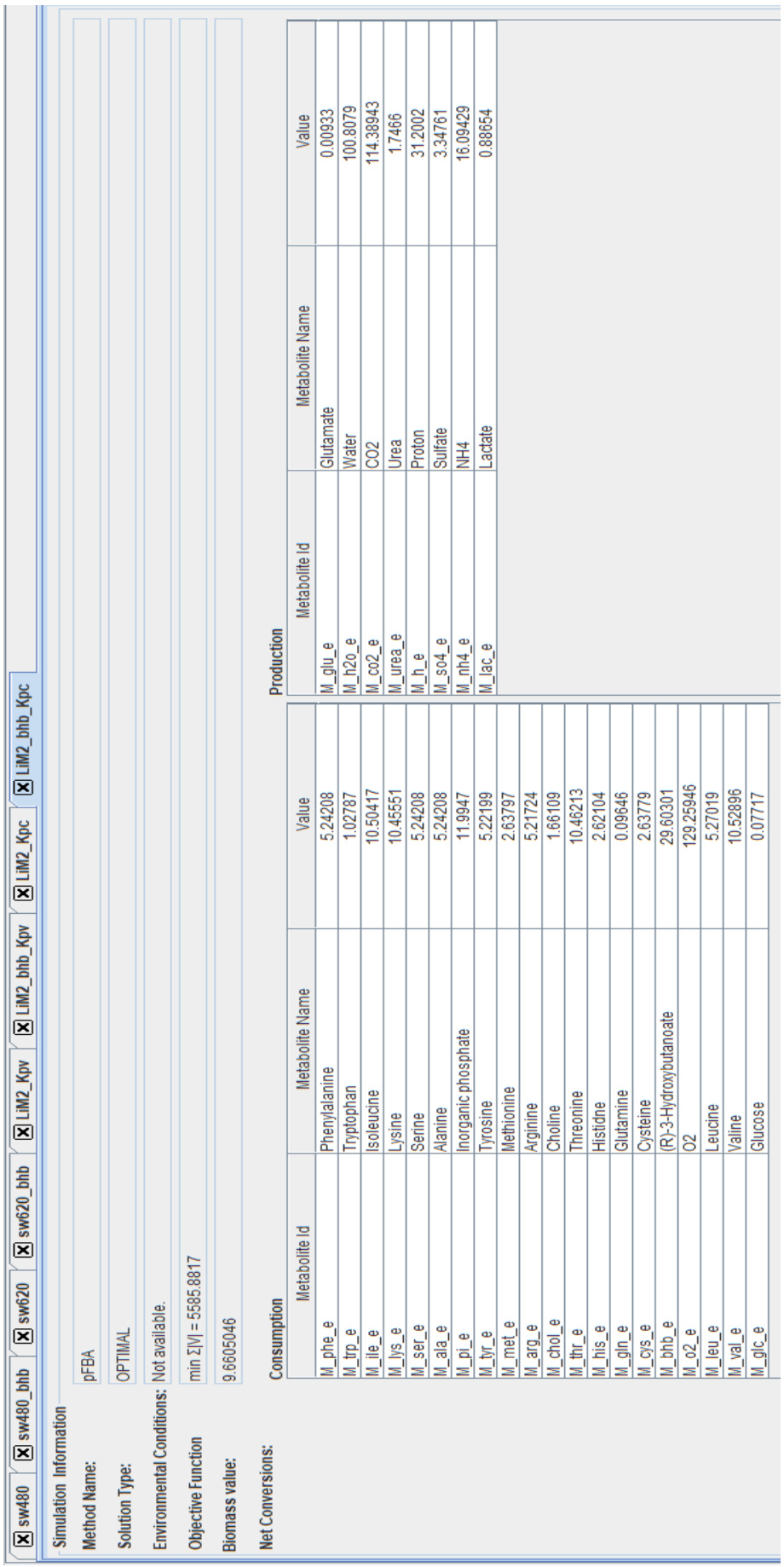
Consumption

Metabolite Id	Metabolite Name	Value
M_phe_e	Phenylalanine	5.94584
M_trp_e	Tryptophan	1.16586
M_ile_e	Isoleucine	11.91438
M_ser_e	Serine	5.94584
M_lys_e	Lysine	11.8592
M_ala_e	Alanine	5.94584
M_tyr_e	Tyrosine	5.92306
M_pi_e	Inorganic phosphate	11.4451
M_met_e	Methionine	2.99287
M_arg_e	Arginine	5.91767
M_chol_e	Choline	1.58497
M_thr_e	Threonine	11.86671
M_his_e	Histidine	2.97292
M_h_e	Proton	17.95537
M_cys_e	Cysteine	2.99192
M_gln_e	Glutamine	0.11958
M_o2_e	O2	131.60672
M_leu_e	Leucine	11.91438
M_val_e	Valine	11.94251
M_glc_e	Glucose	0.2088

Production

Metabolite Id	Metabolite Name	Value
M_glu_e	Glutamate	0.00904
M_co2_e	CO2	111.91398
M_urea_e	Urea	2.60606
M_h2o_e	Water	62.20768
M_so4_e	Sulfate	4.14499
M_nh4_e	NH4	40.12255
M_lac_e	Lactate	1.40832

8.



sw480 | sw480_bhb | sw620 | sw620_bhb | LiM2_Kpv | LiM2_bhb_Kpv | LiM2_Kpc | LiM2_bhb_Kpc

Simulation Information

Method Name: pFBA

Solution Type: OPTIMAL

Environmental Conditions: Not available.

Objective Function min Σ|v| = 5585.8817

Biomass value: 9.6605046

Net Conversions:

Consumption

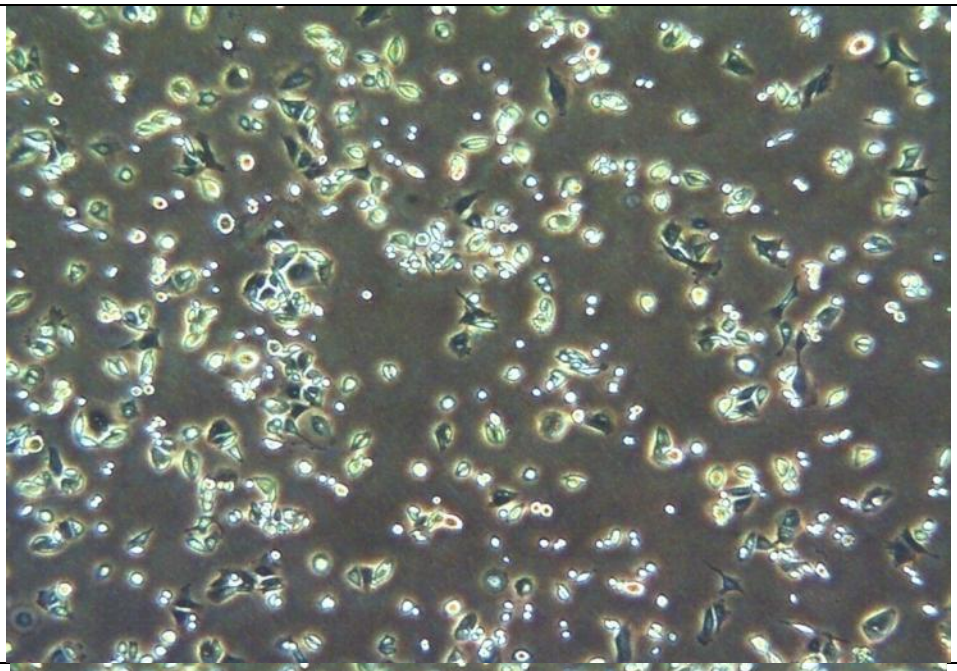
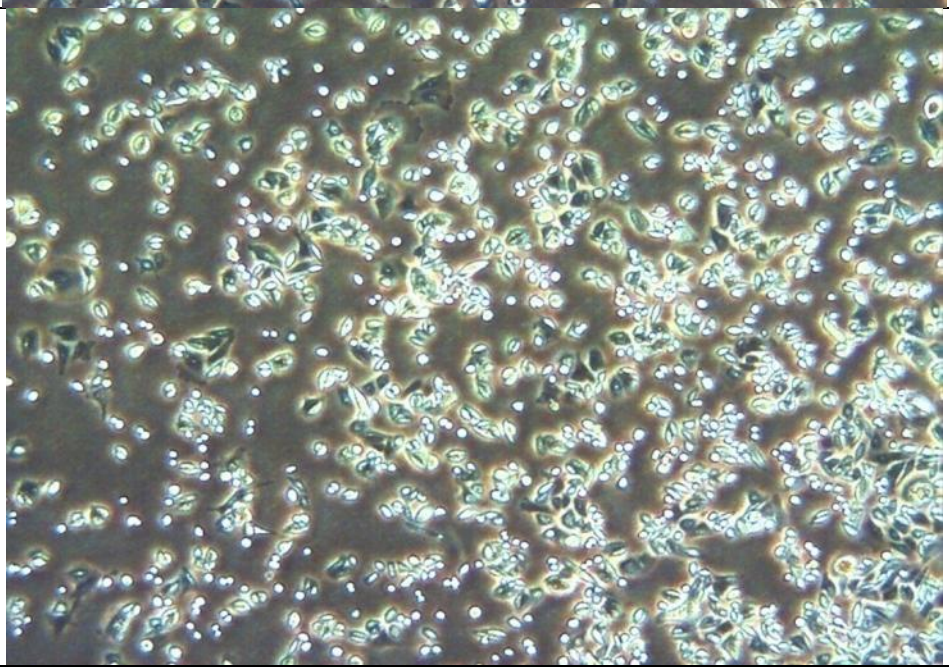
Metabolite Id	Metabolite Name	Value
M_phe_e	Phenylalanine	5.24208
M_trp_e	Tryptophan	1.02787
M_ile_e	Isoleucine	10.50417
M_lys_e	Lysine	10.45551
M_ser_e	Serine	5.24208
M_ala_e	Alanine	5.24208
M_pi_e	Inorganic phosphate	11.9947
M_tyr_e	Tyrosine	5.22199
M_met_e	Methionine	2.63797
M_arg_e	Arginine	5.21724
M_chol_e	Choline	1.66109
M_thr_e	Threonine	10.46213
M_his_e	Histidne	2.62104
M_gln_e	Glutamine	0.09646
M_cys_e	Cysteine	2.63779
M_bhb_e	(R)-3-Hydroxybutanoate	29.60301
M_o2_e	O2	129.25946
M_leu_e	Leucine	5.27019
M_val_e	Valine	10.52896
M_glc_e	Glucose	0.07717

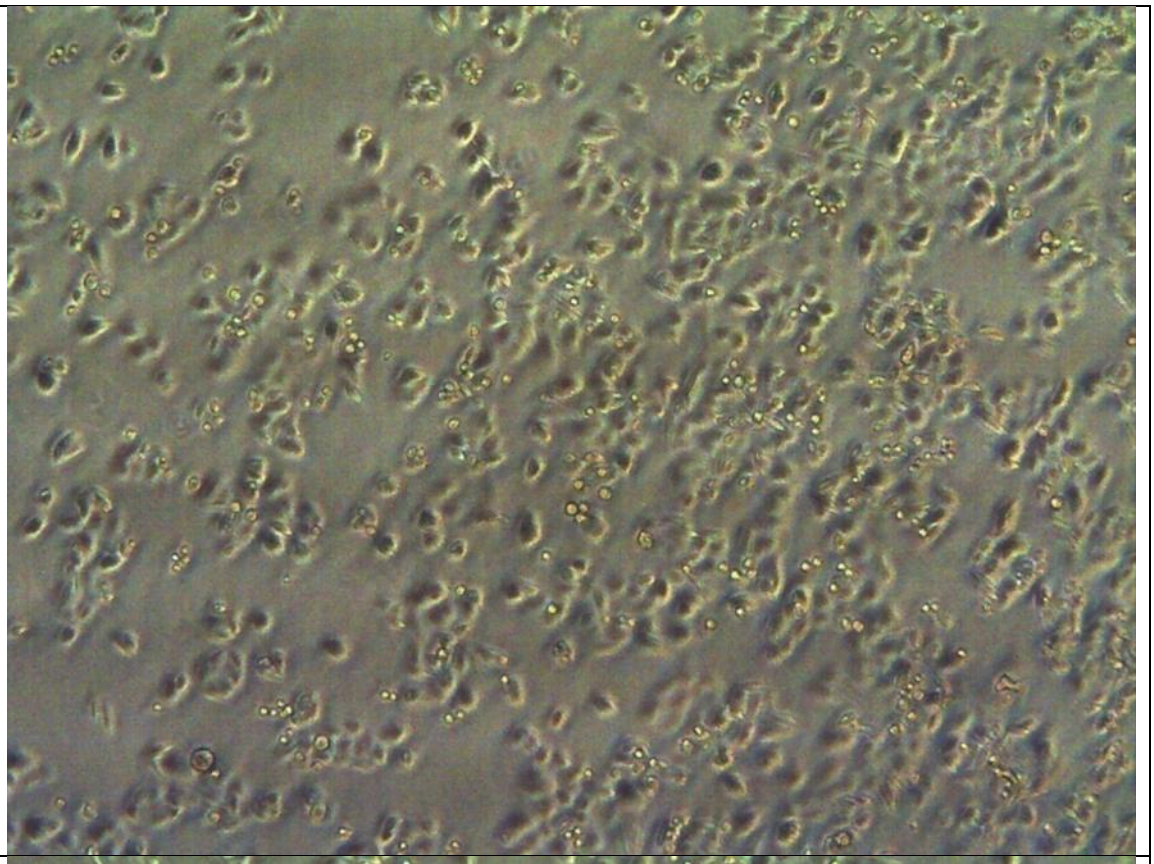
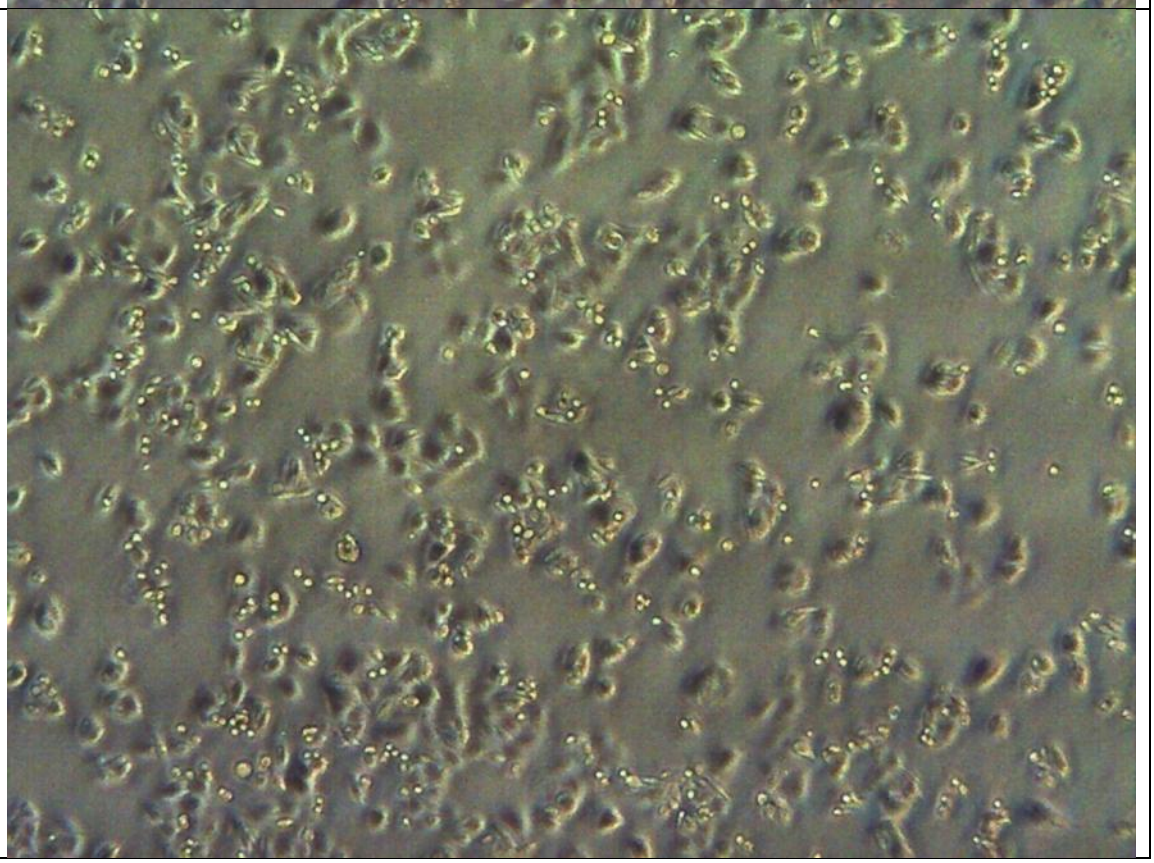
Production

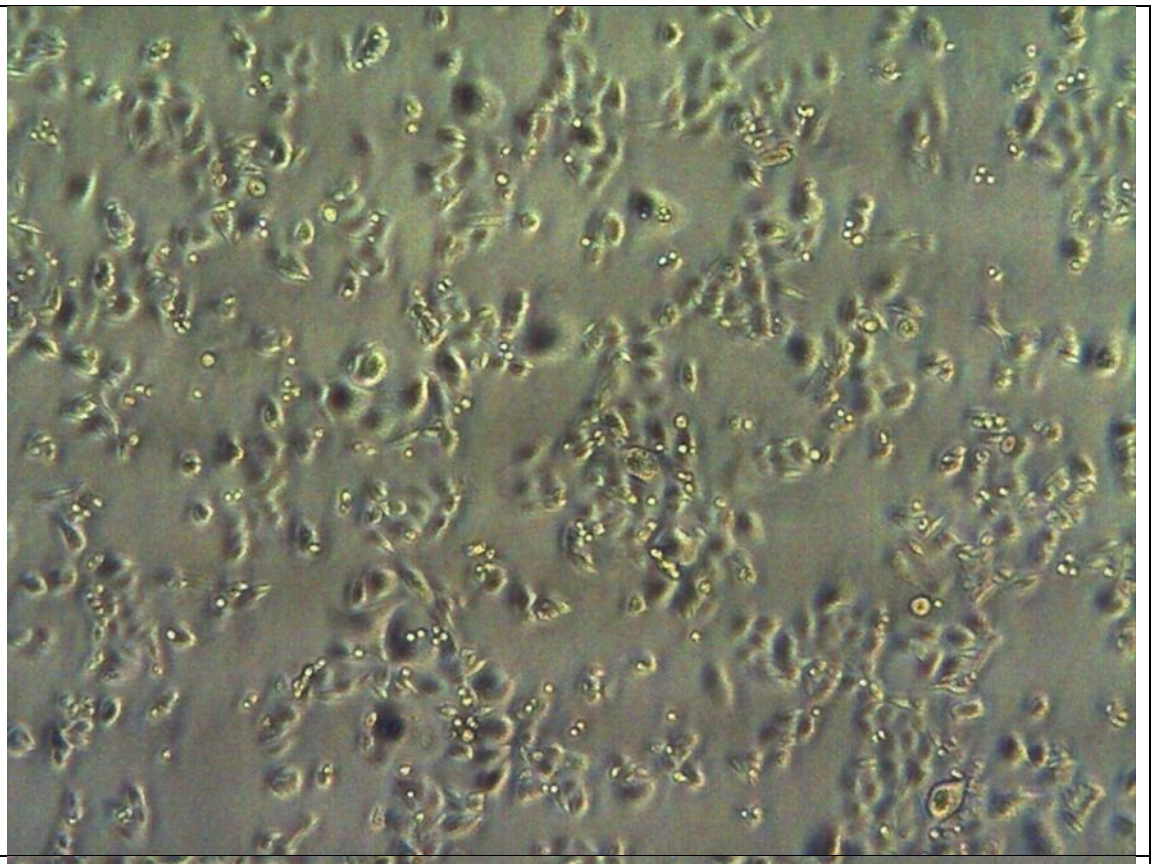
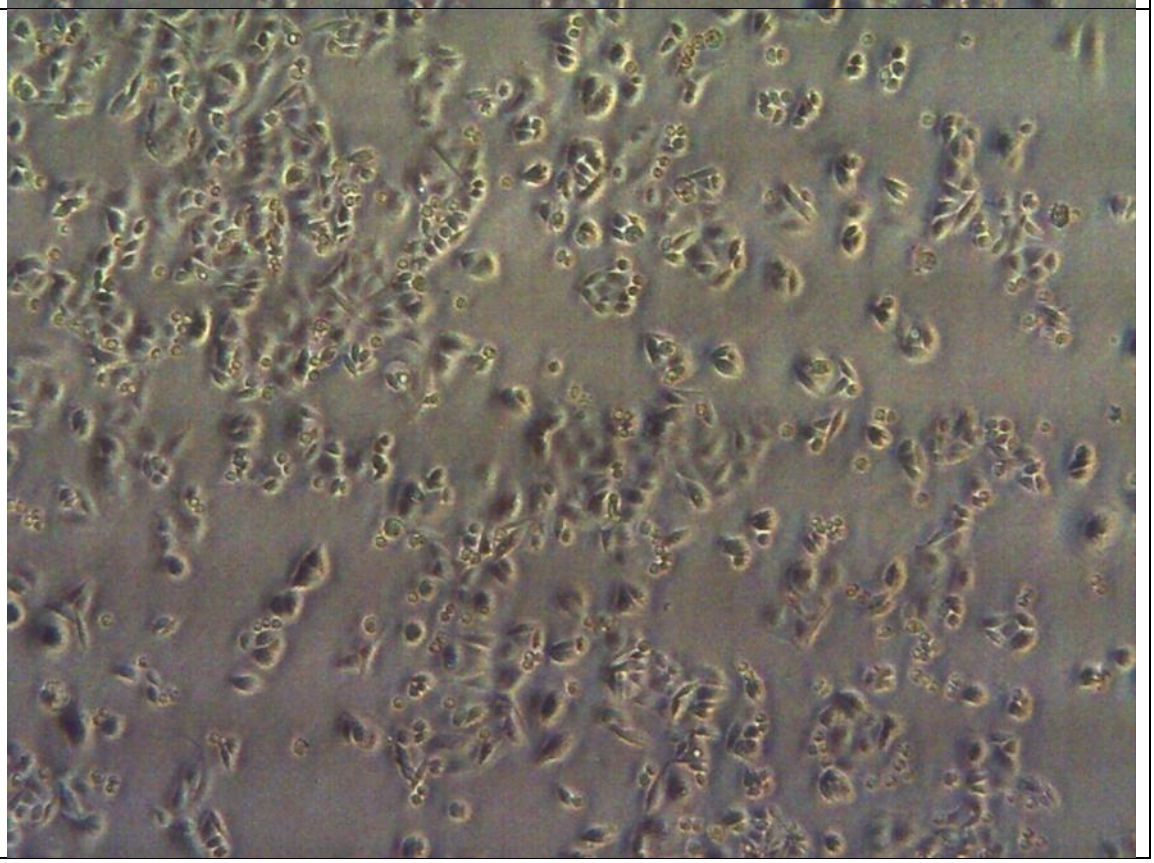
Metabolite Id	Metabolite Name	Value
M_glu_e	Glutamate	0.00933
M_h2o_e	Water	100.8079
M_co2_e	CO2	114.38943
M_urea_e	Urea	1.7466
M_h_e	Proton	31.2002
M_so4_e	Sulfate	3.34761
M_nh4_e	NH4	16.09429
M_lac_e	Lactate	0.88654

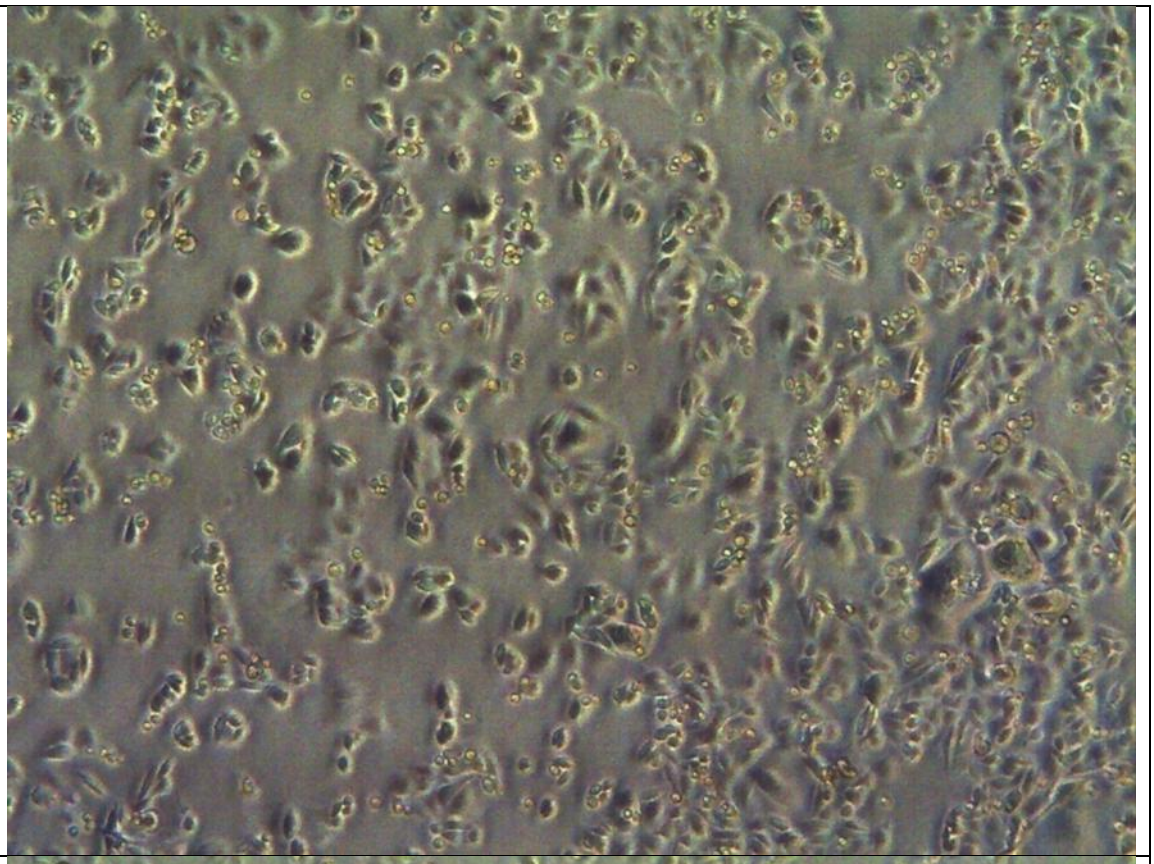
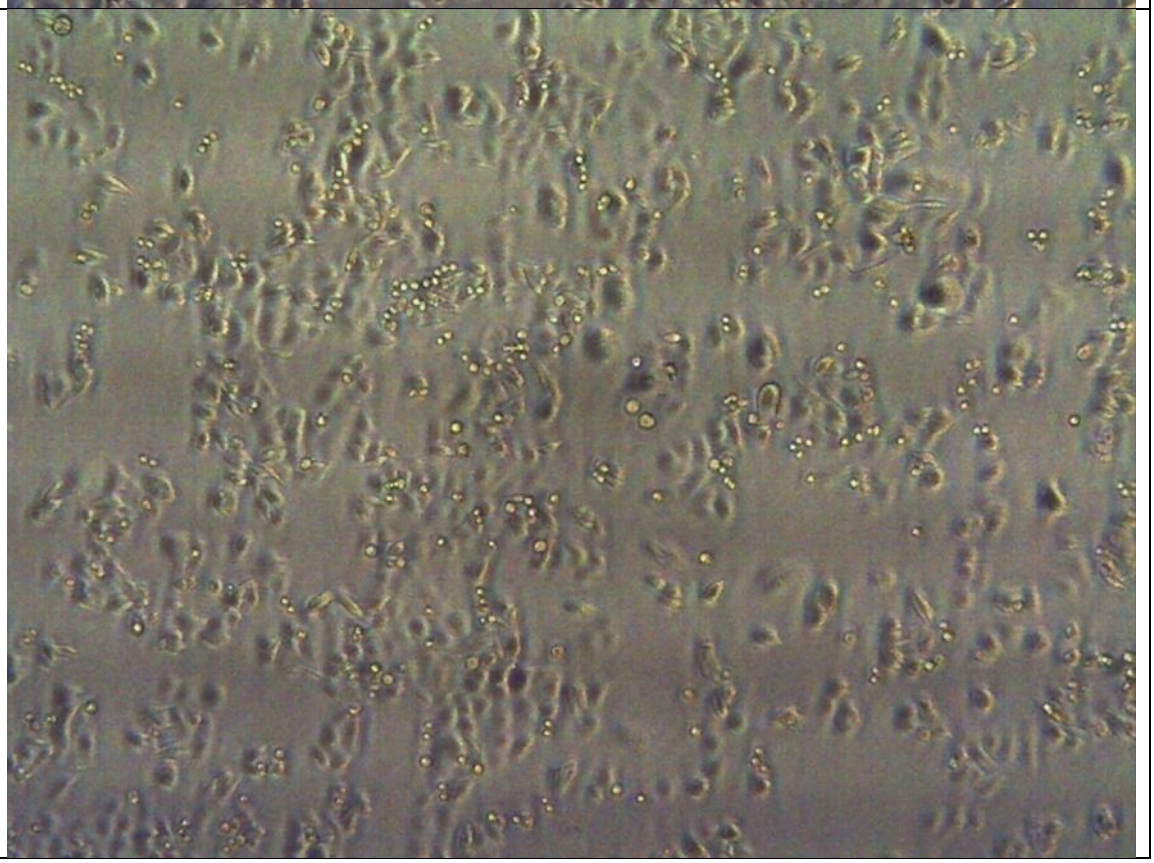
ANNEX X: Cell Pictures

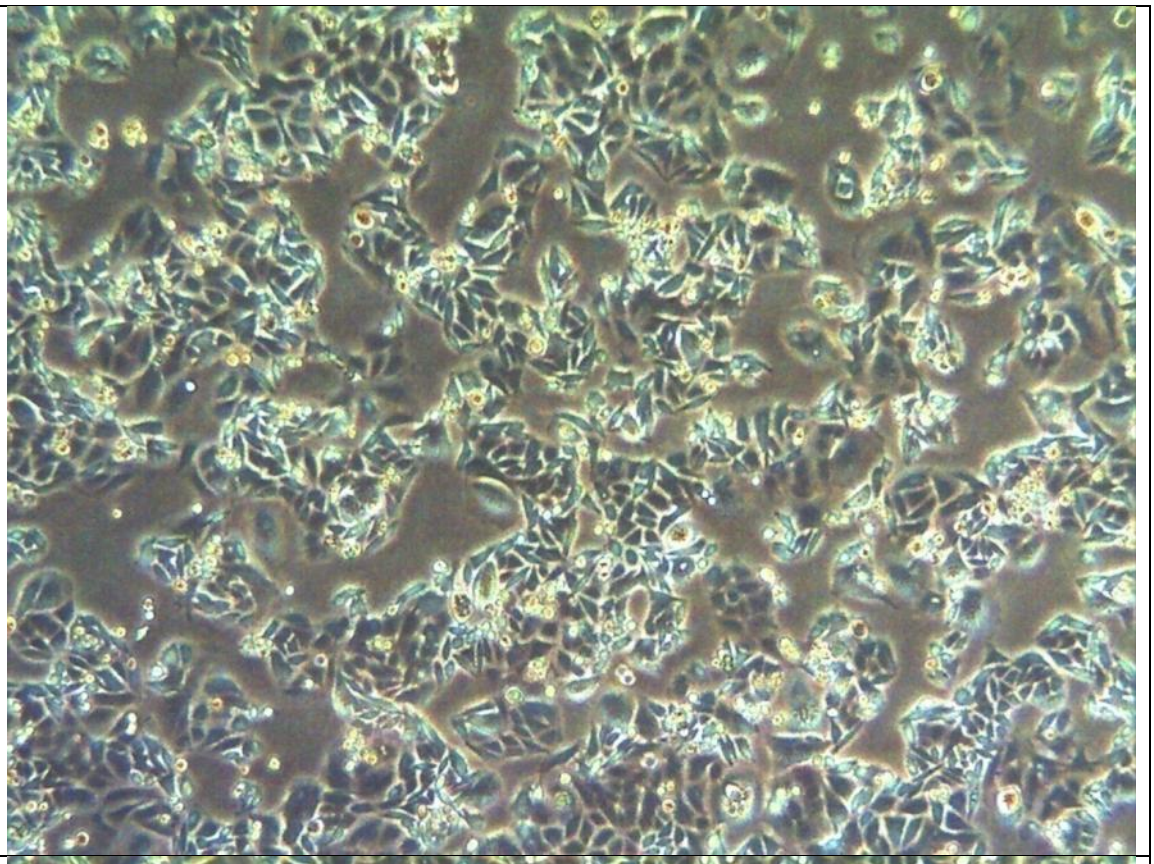
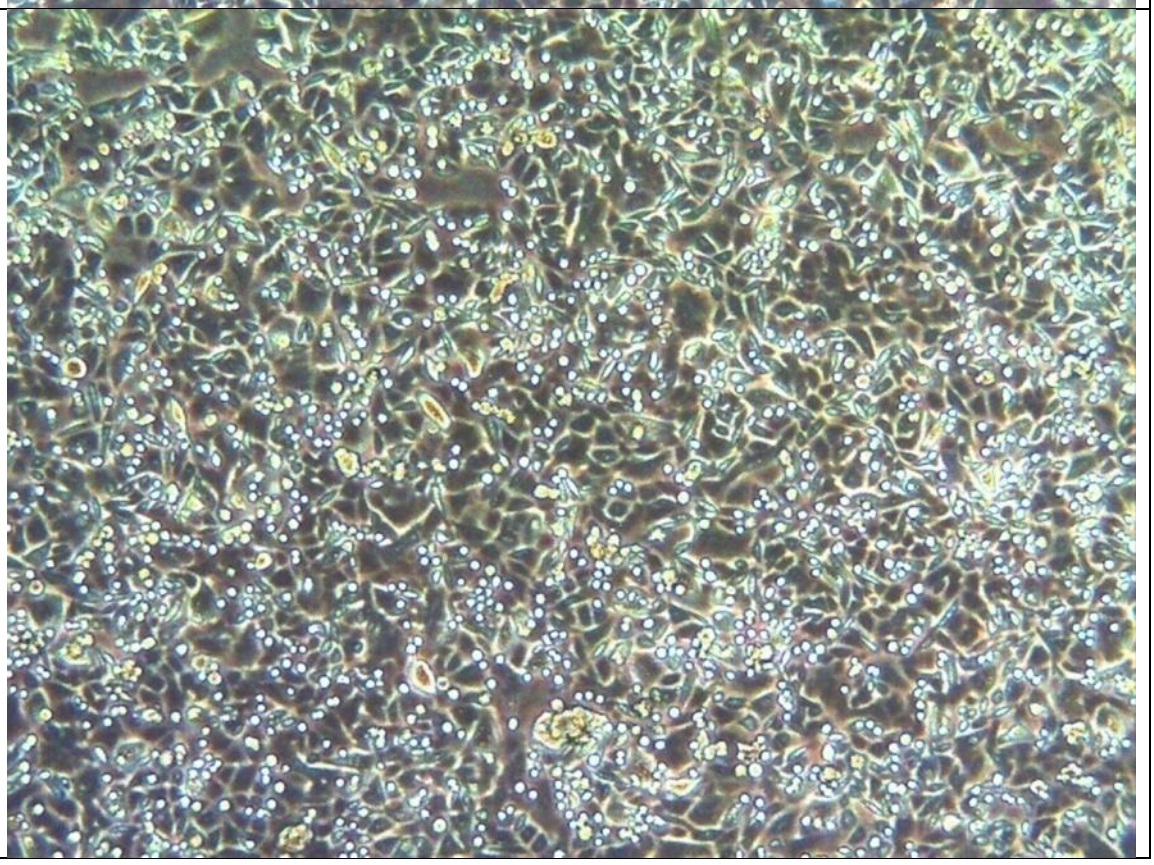
These are the pictures taken with the microscope before the counting. They are sorted by cell line (480, 620, LiM2), time with the medium of study when the picture was taken (0, 24 and 48 hours), the number of the well (1, 2 or 3) and the magnification of the picture (40x and 100x).

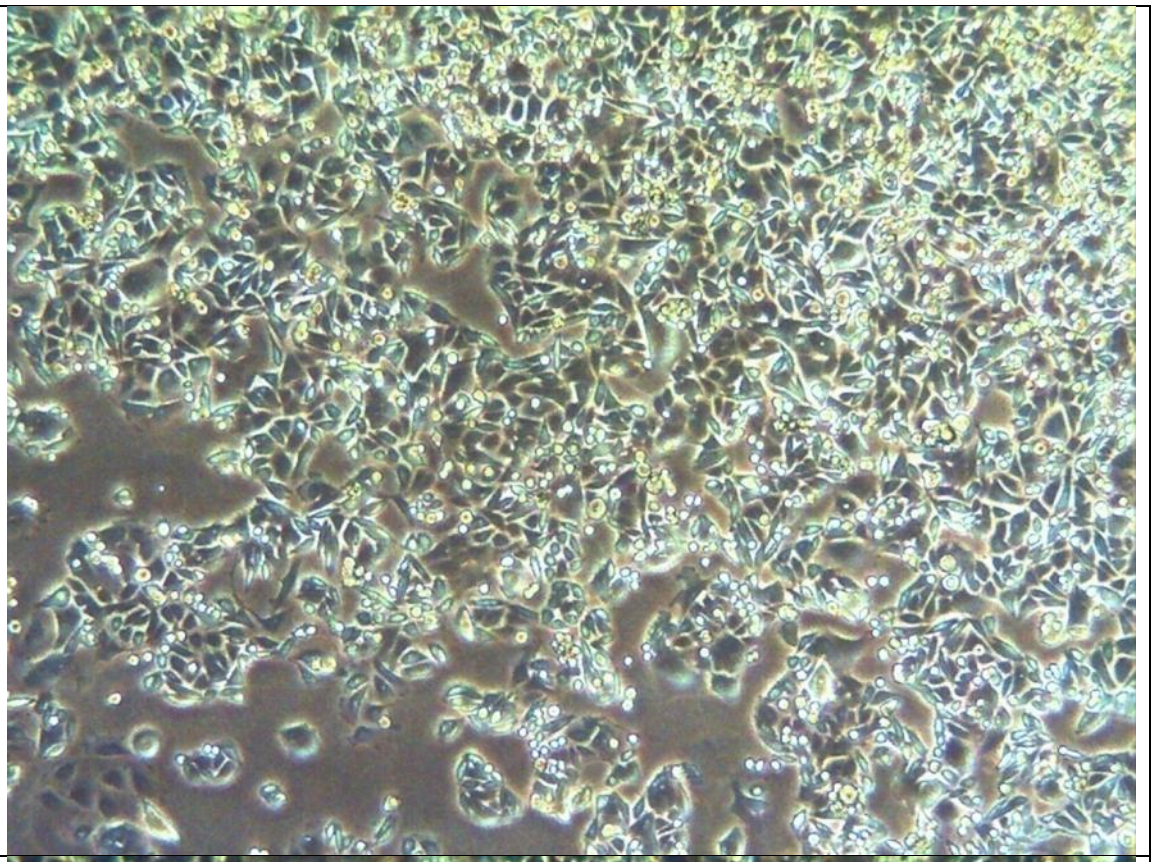
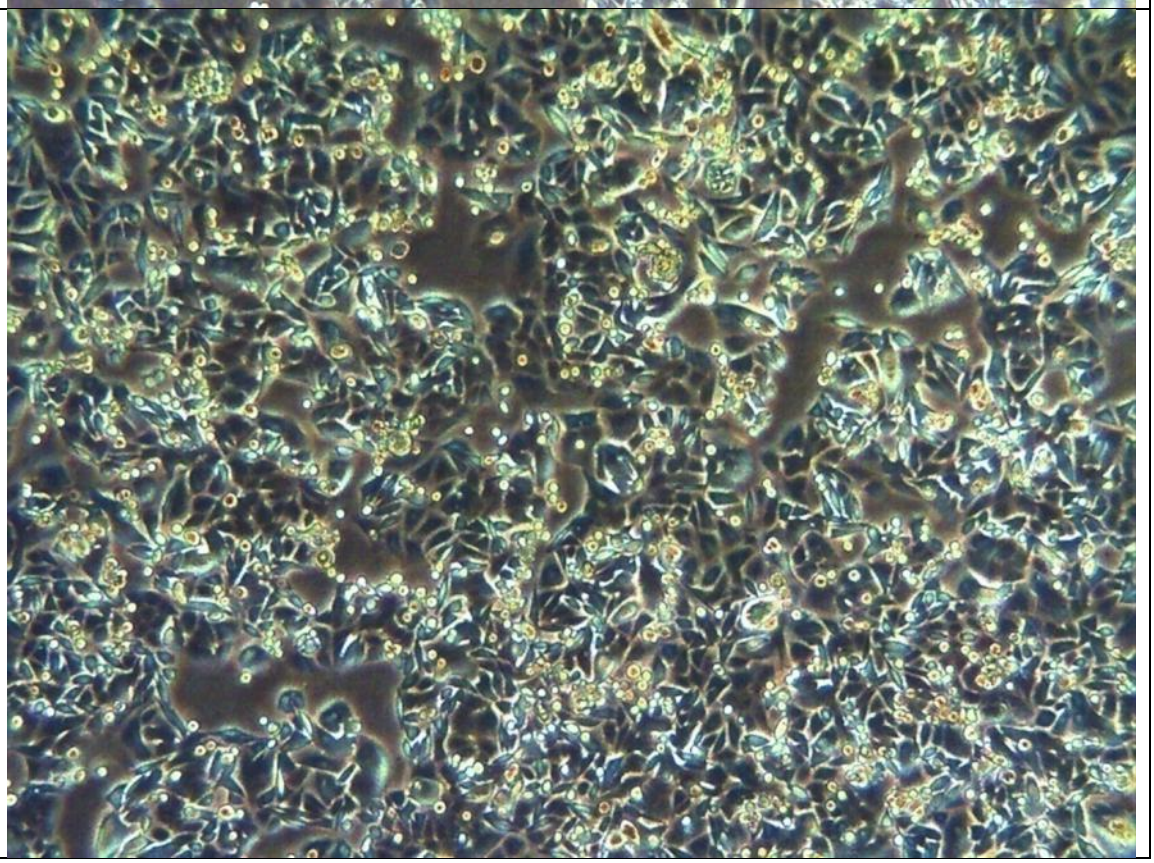
	40x
480 0h 1	
480 0h 2	

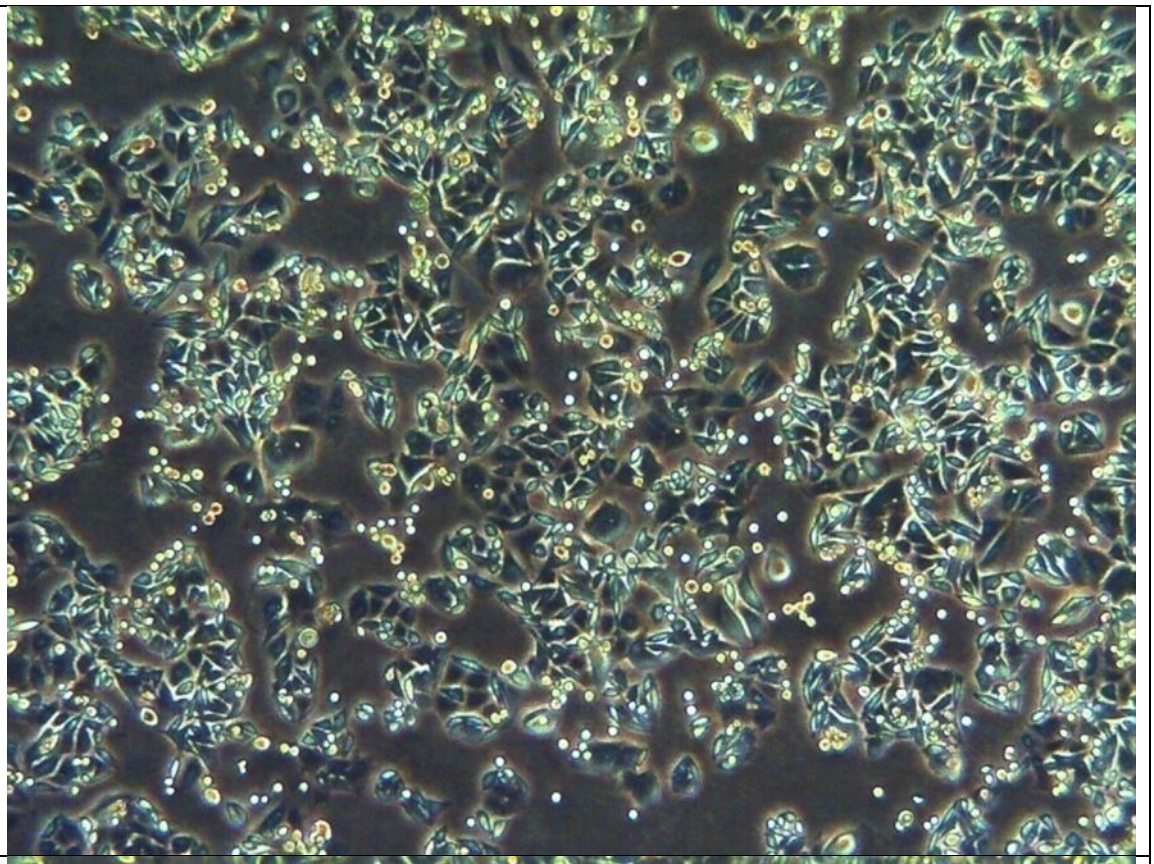
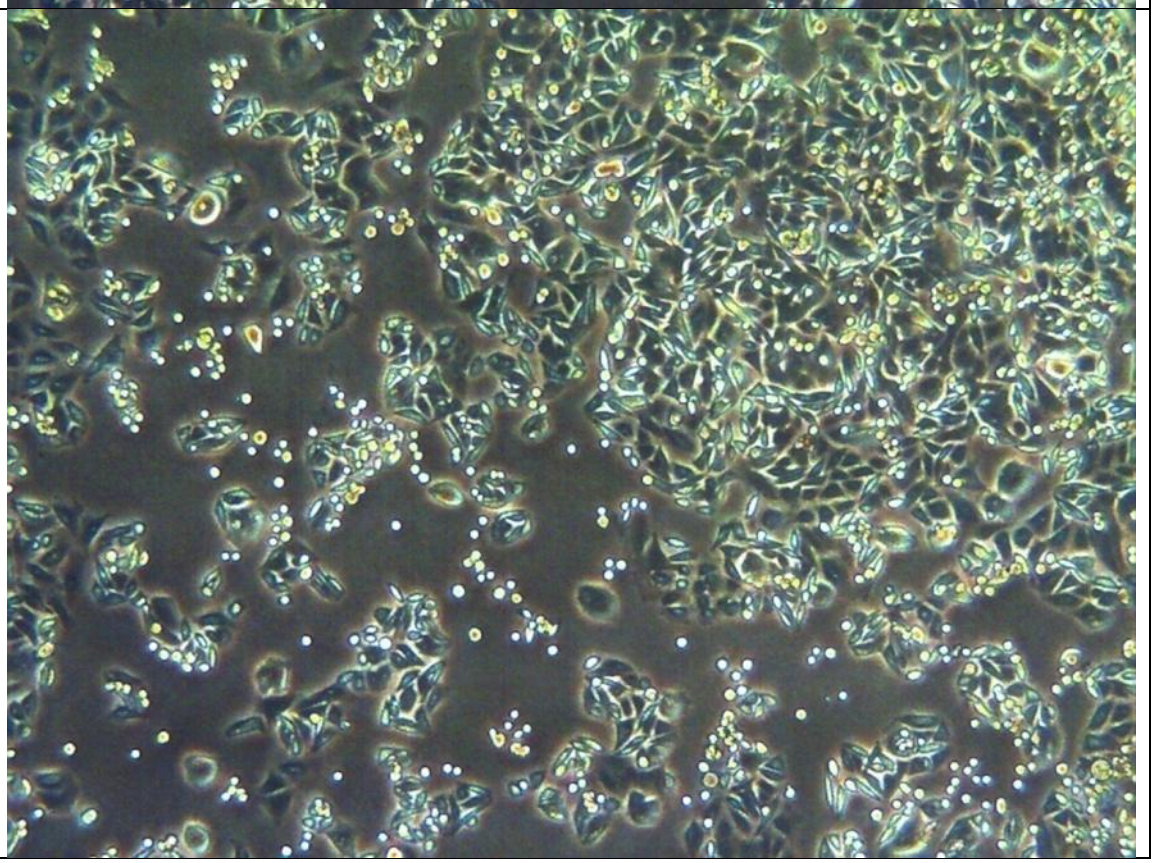
480 24h Cntlr 1	
480 24h Cntlr 2	

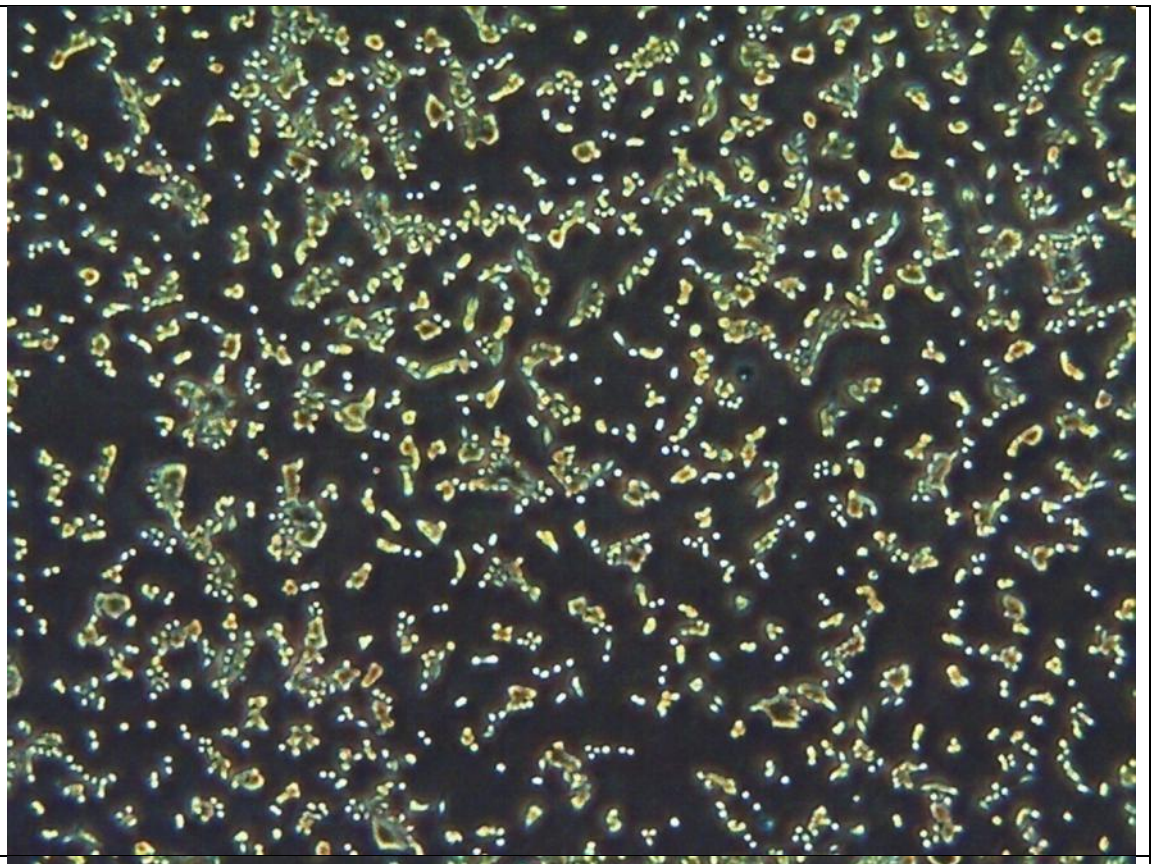
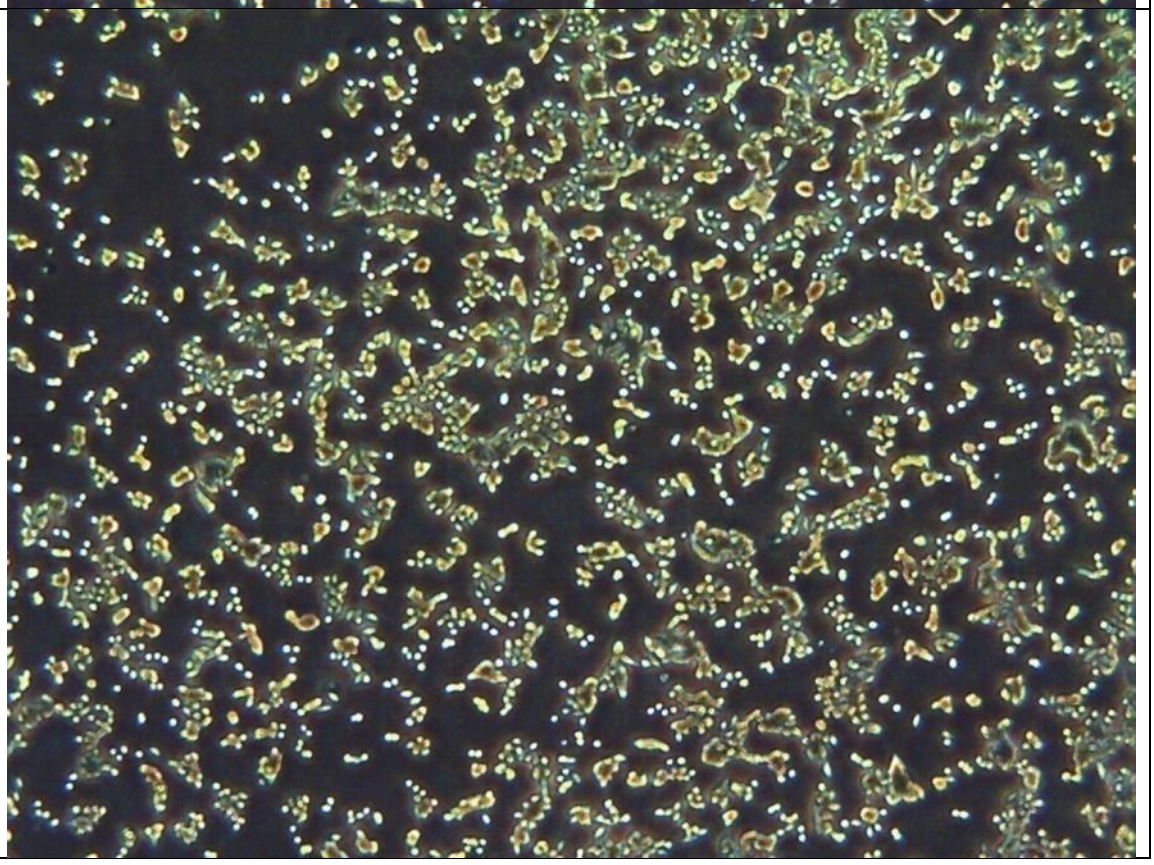
480 24h Cntlr 3	
480 24h Bhb 1	

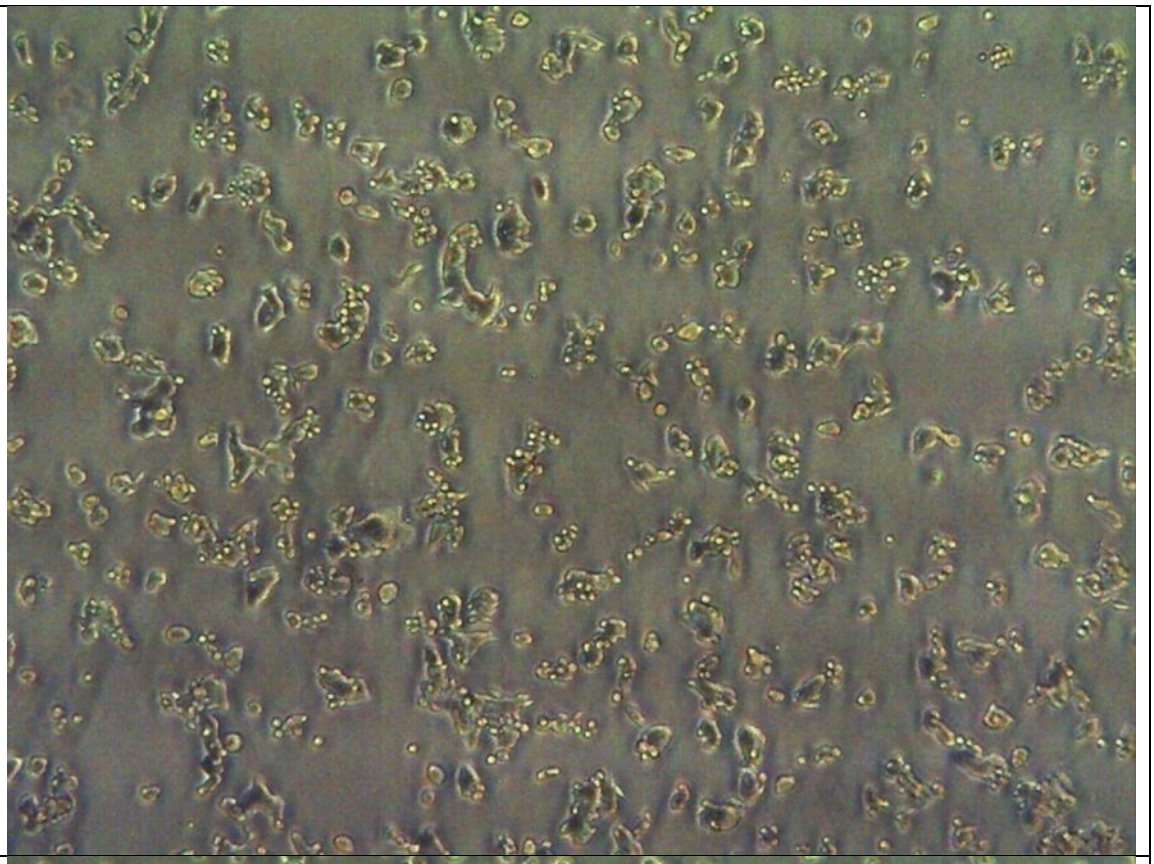
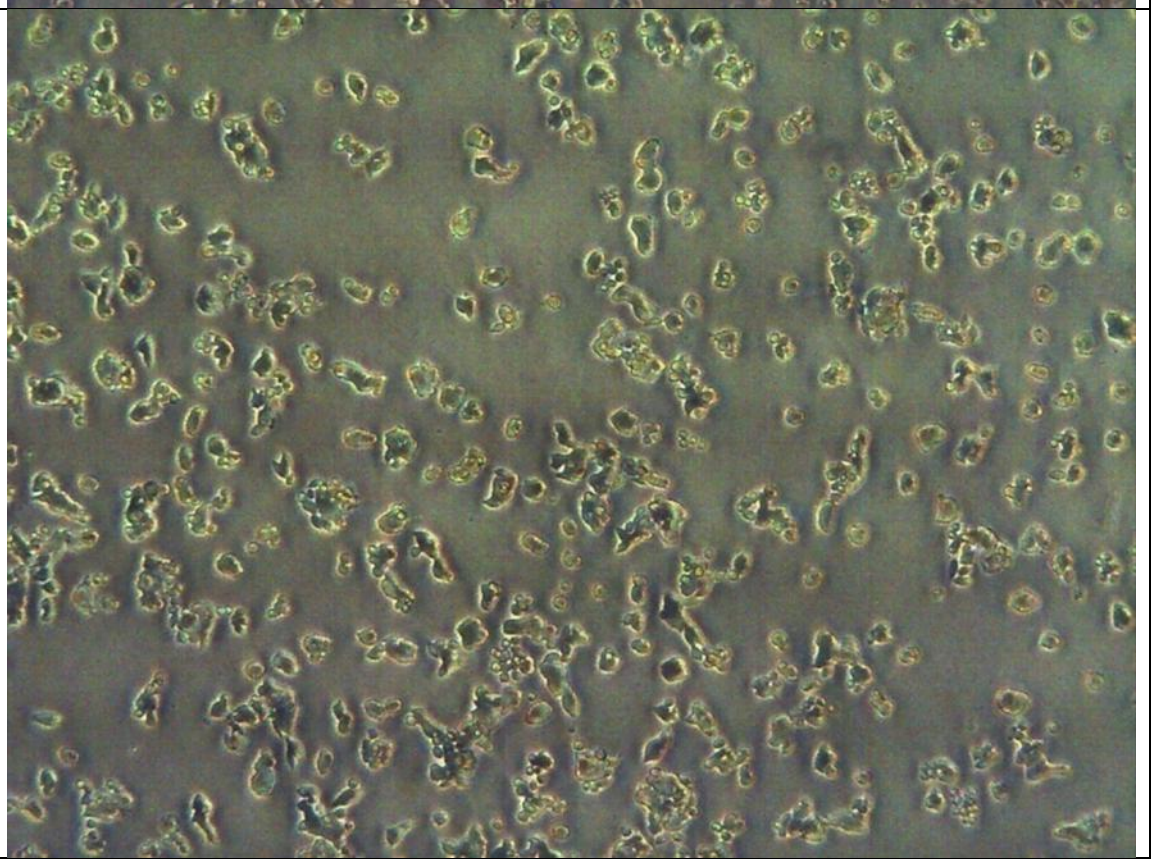
480 24h Bhb 2	
480 24h Bhb 3	

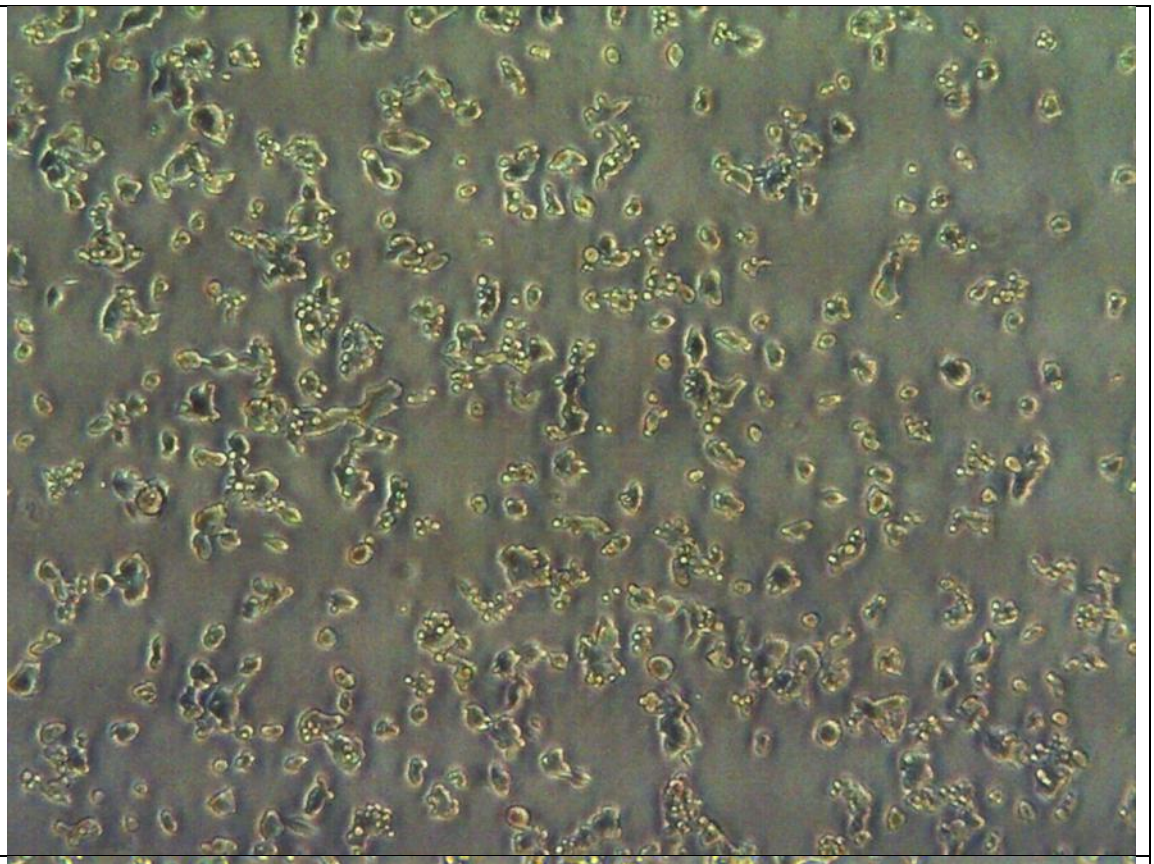
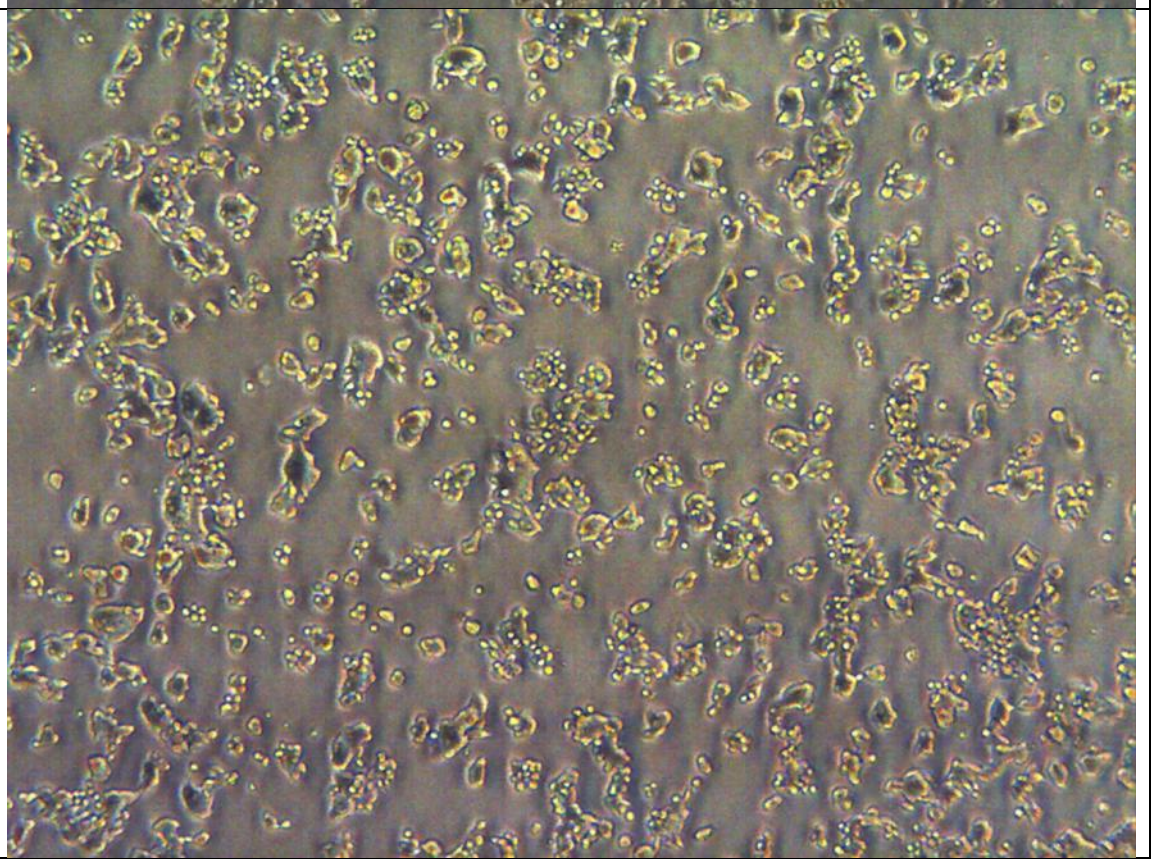
480 48h Cntlr 1	
480 48h Cntlr 2	

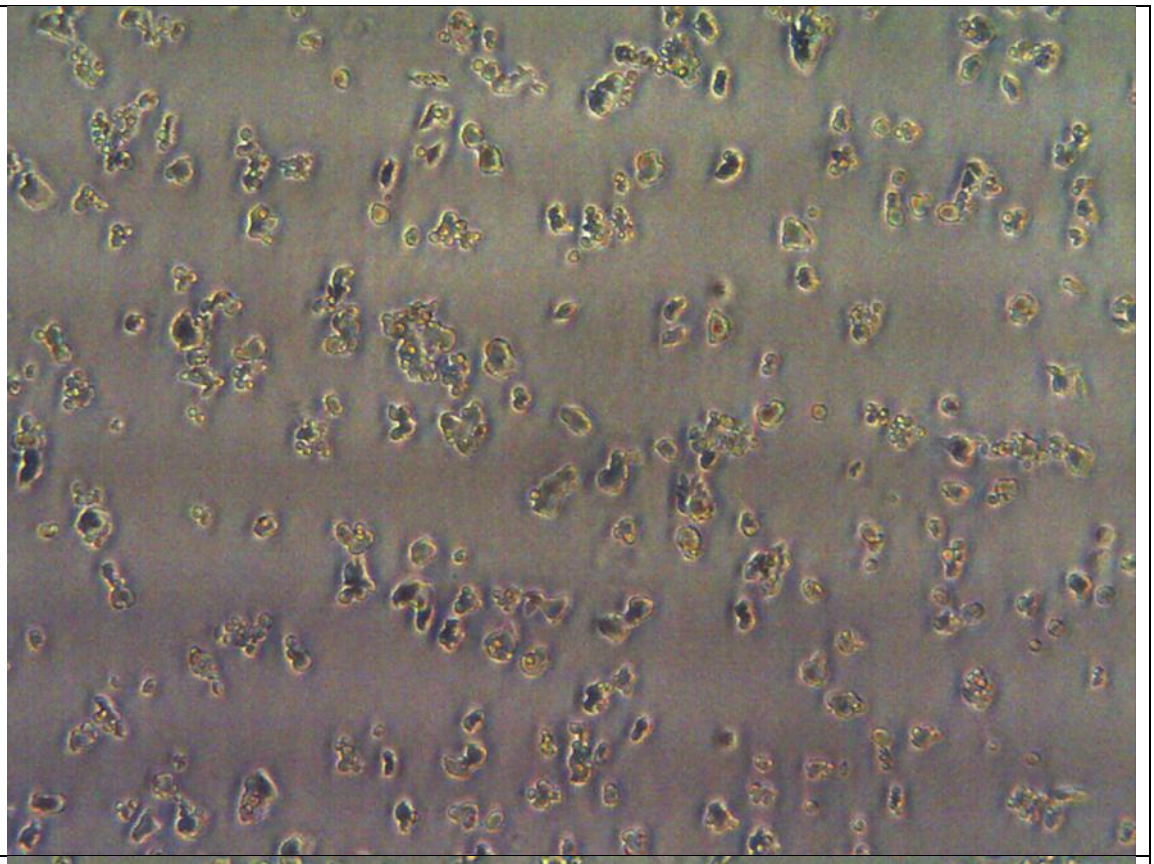
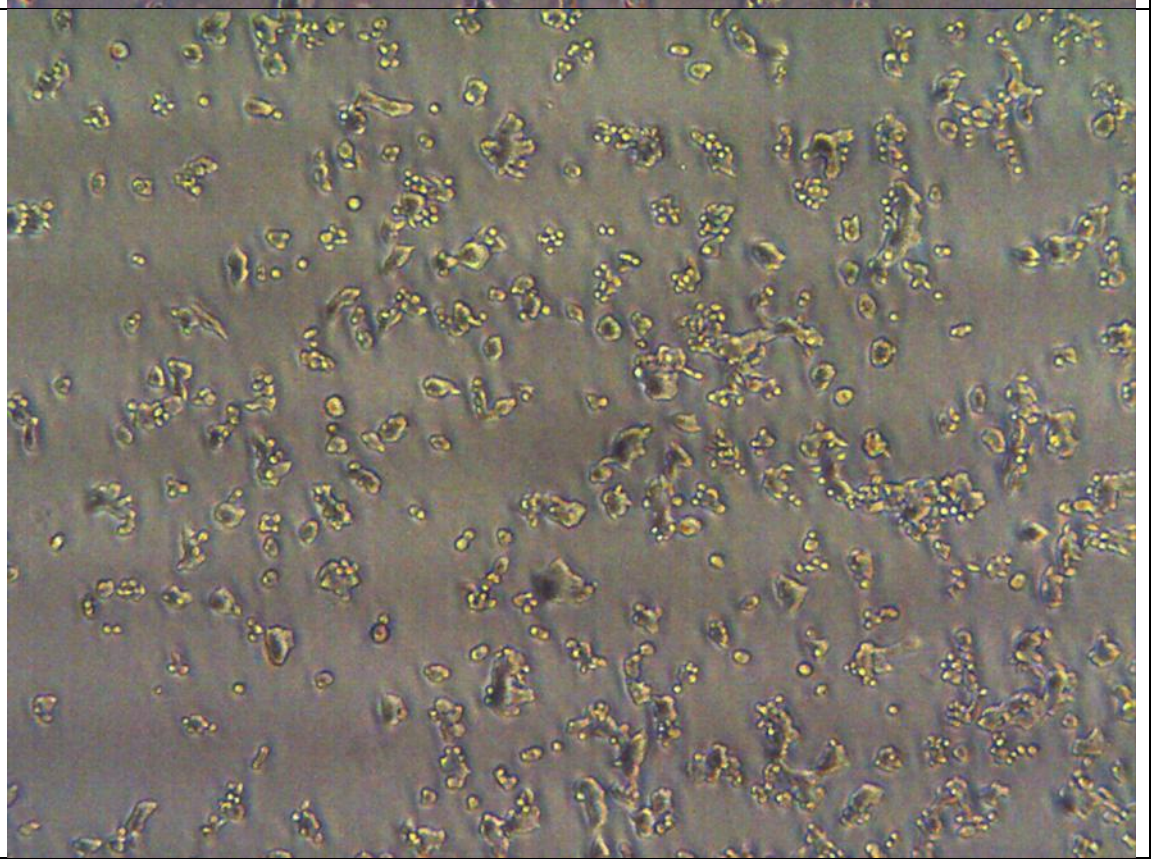
480 48h Cntlr 3	
480 48h Bhb 1	

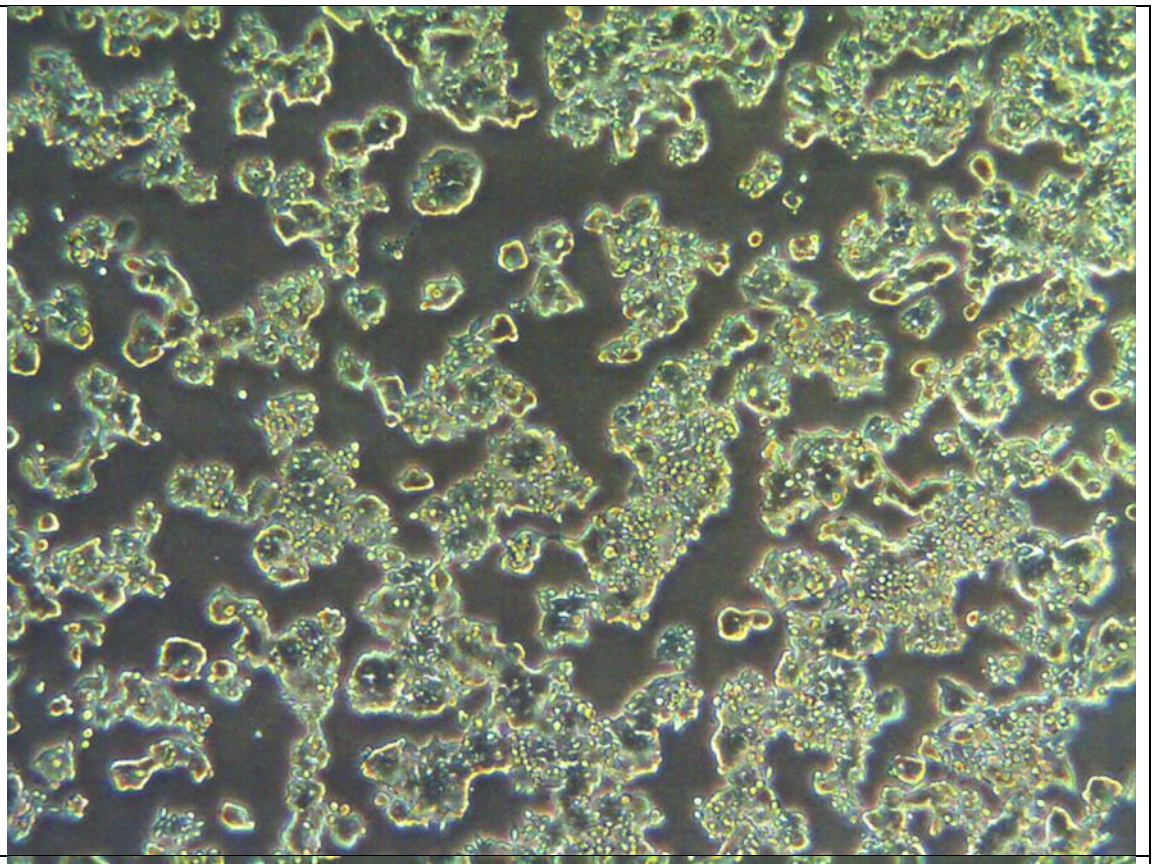
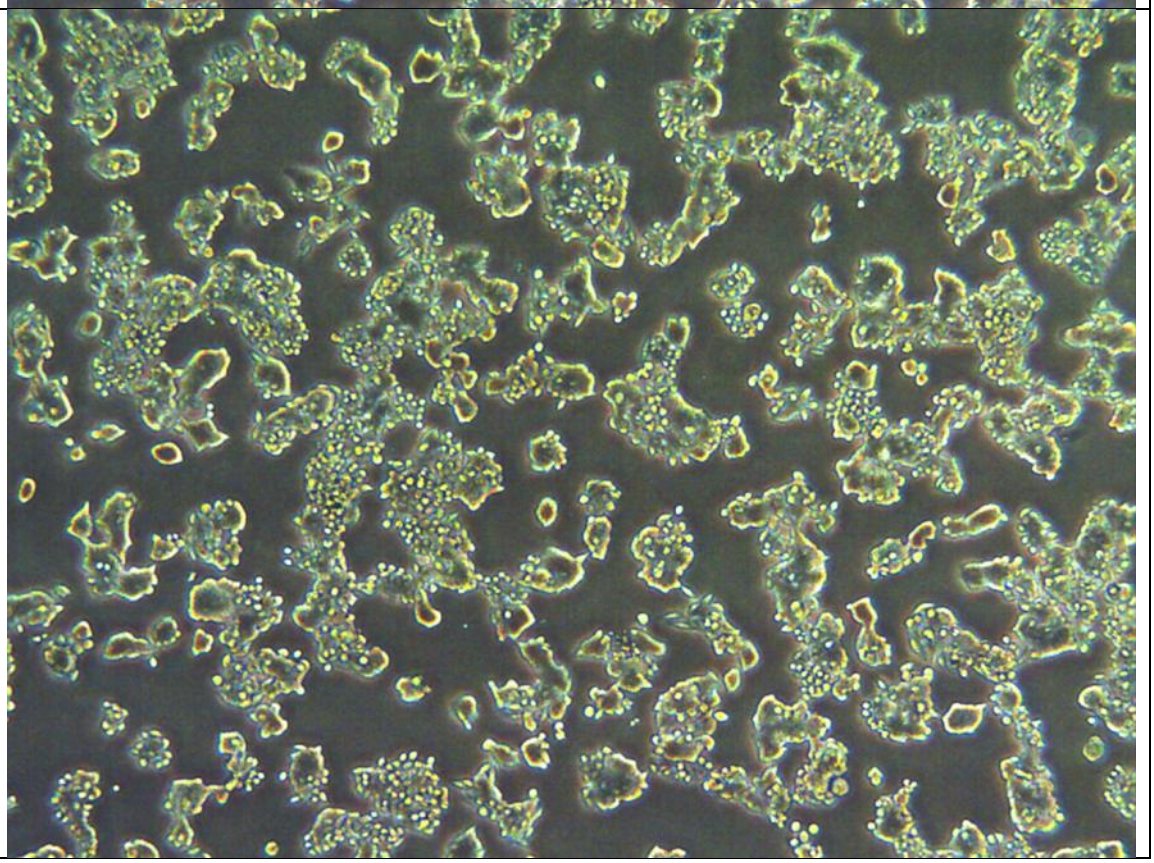
480 48h Bhb 2	
480 48h Bhb 3	

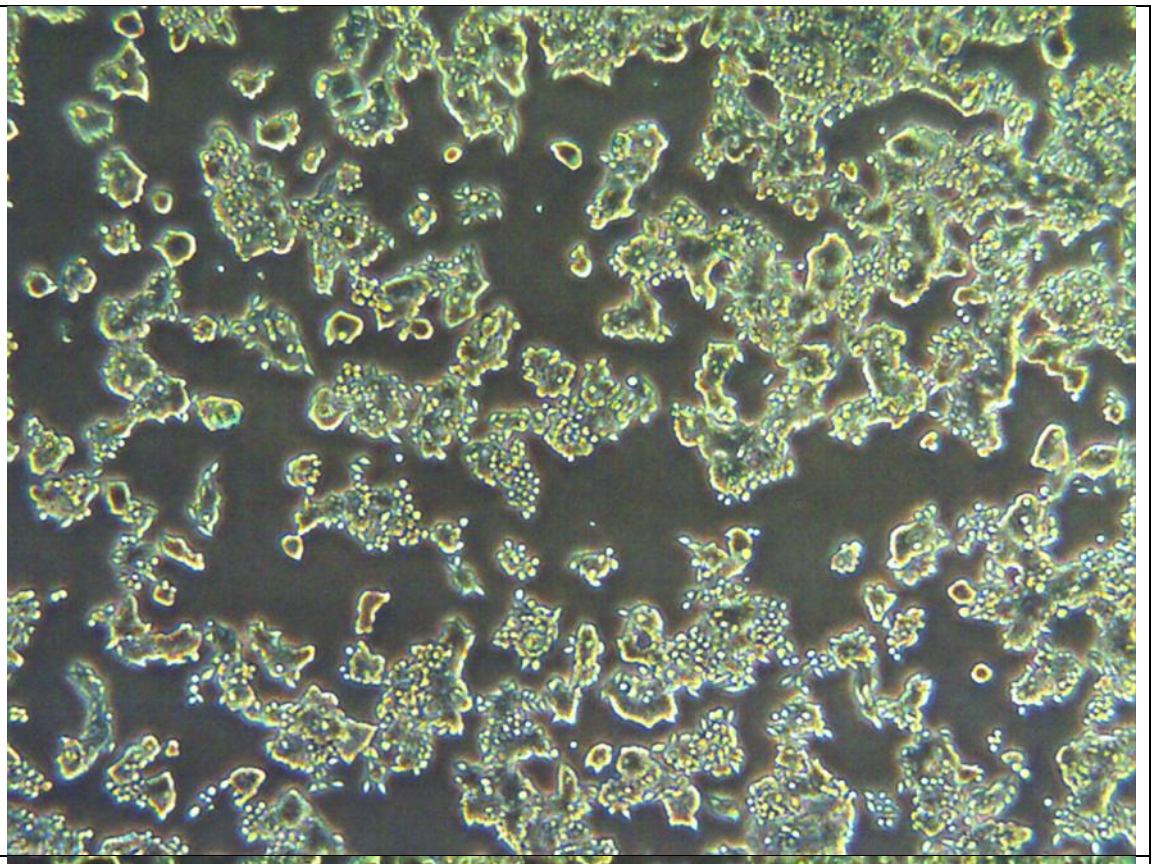
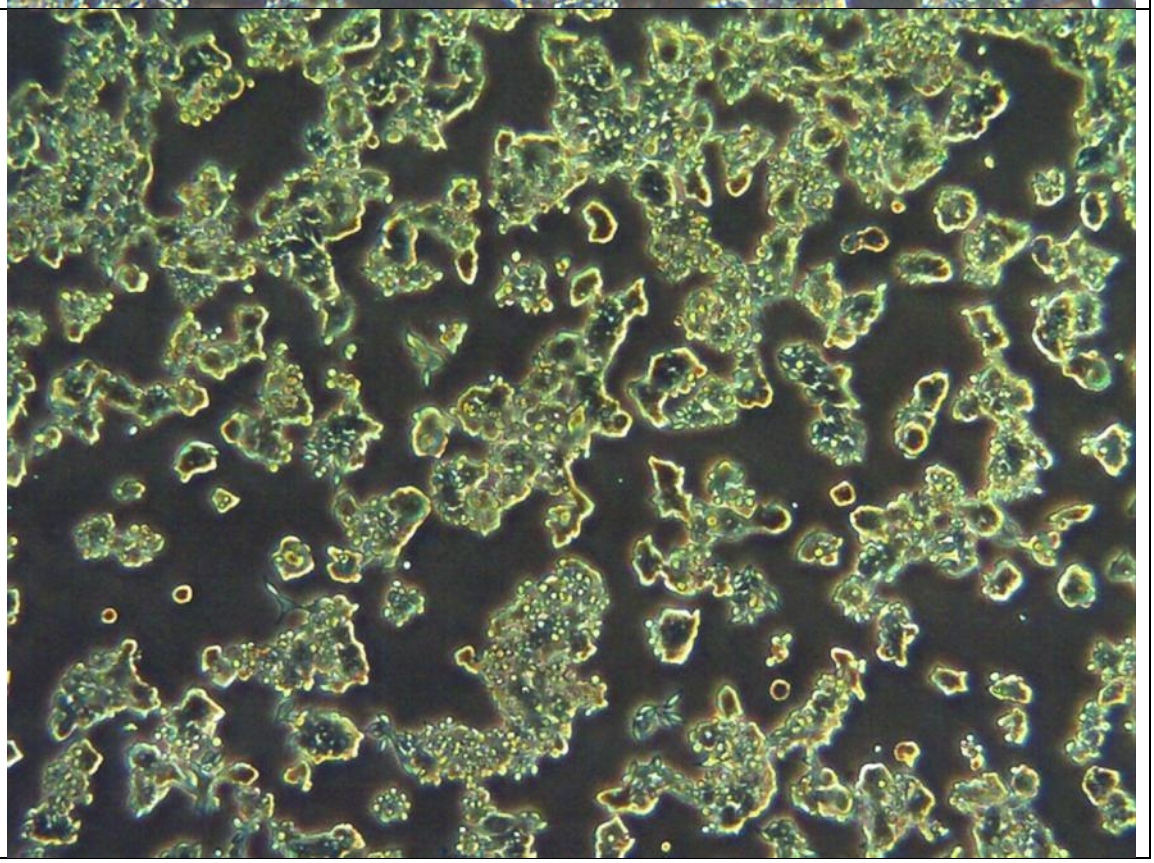
6200h1	
6200h2	

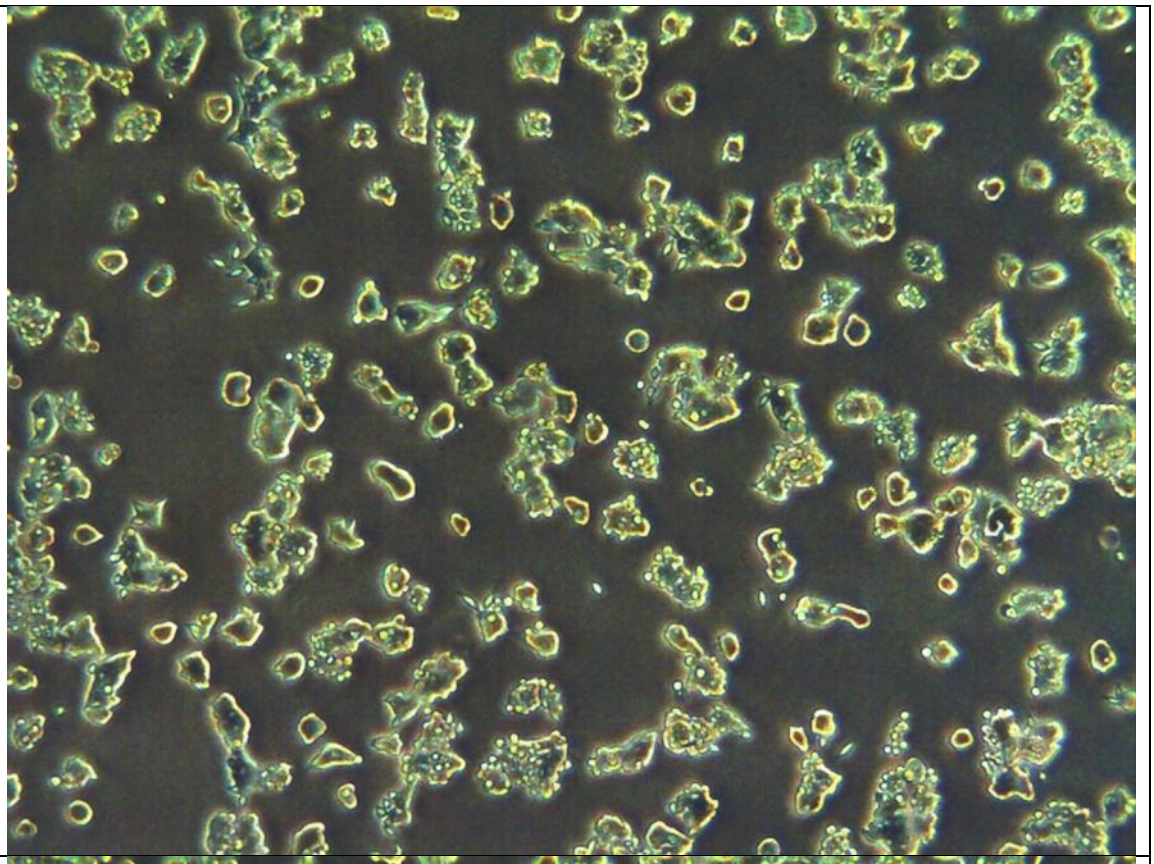
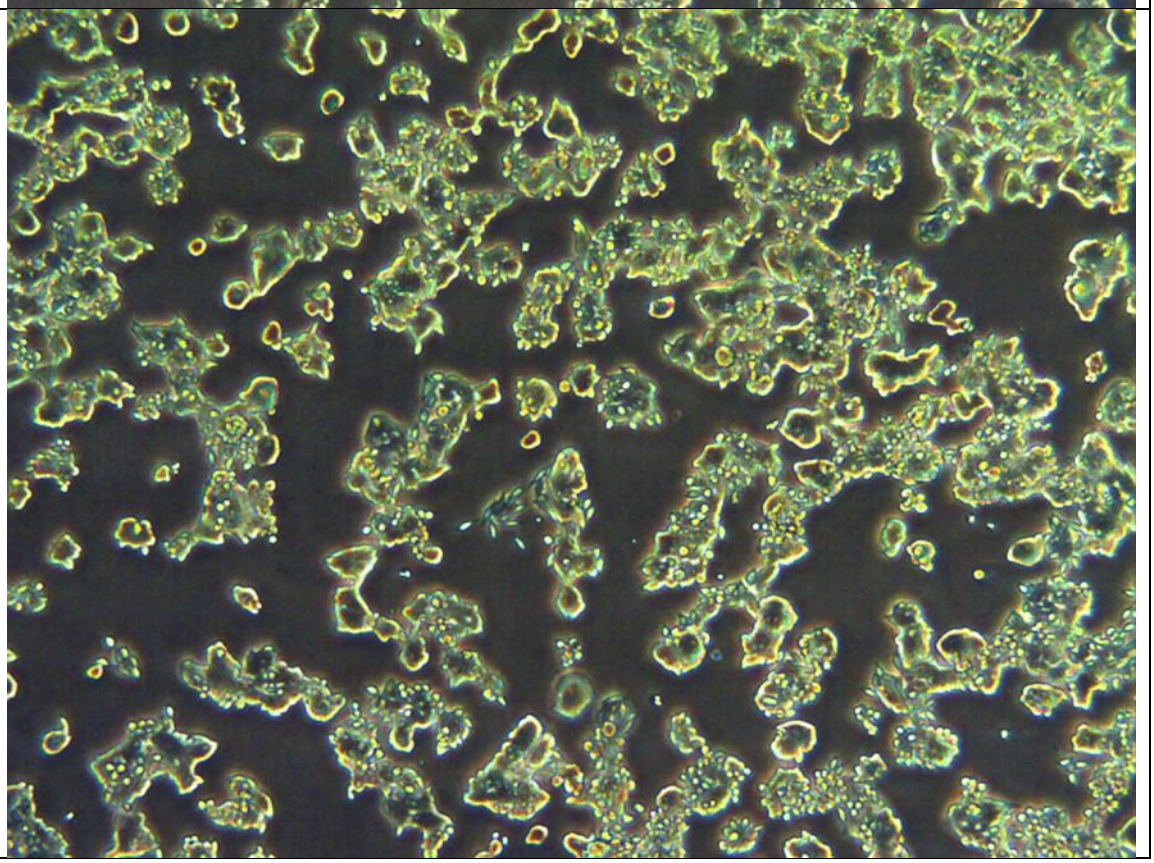
620 24h Cntlr 1	
620 24h Cntlr 2	

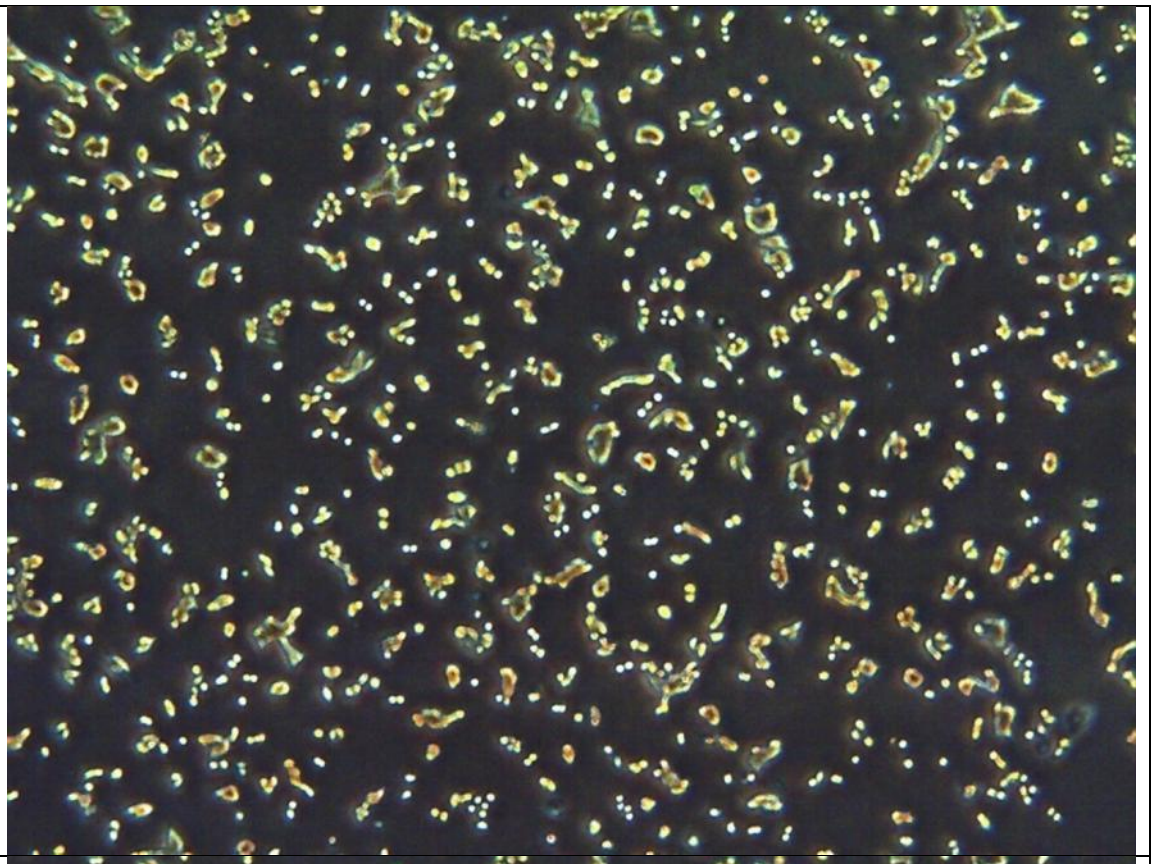
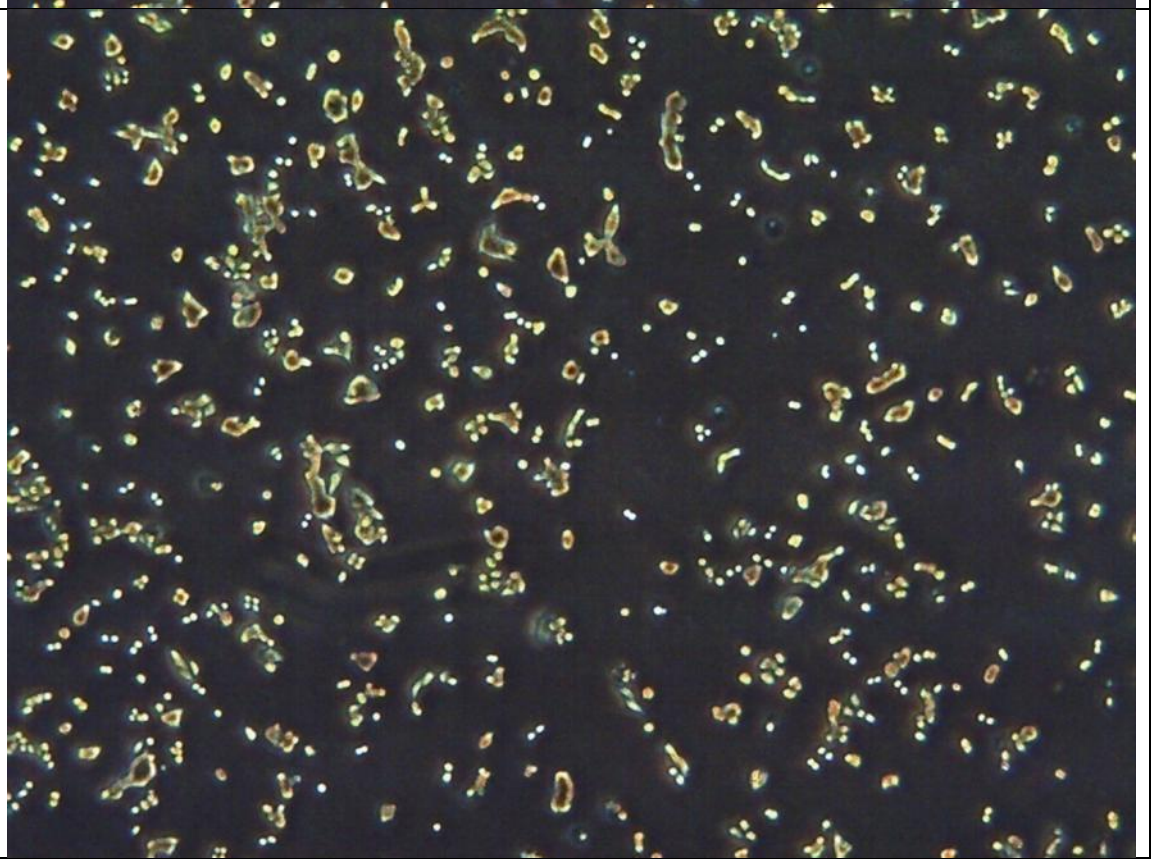
620 24h Cntlr 3	
620 24h Bhb 1	

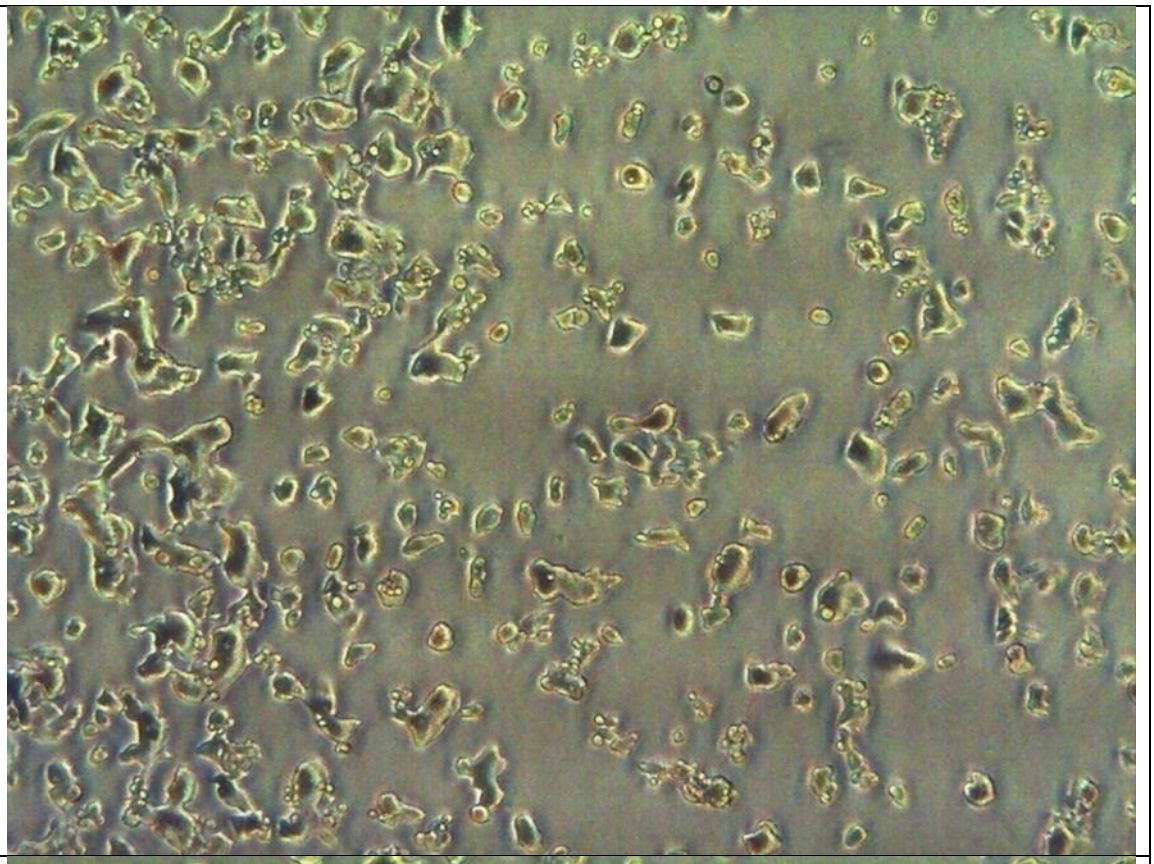
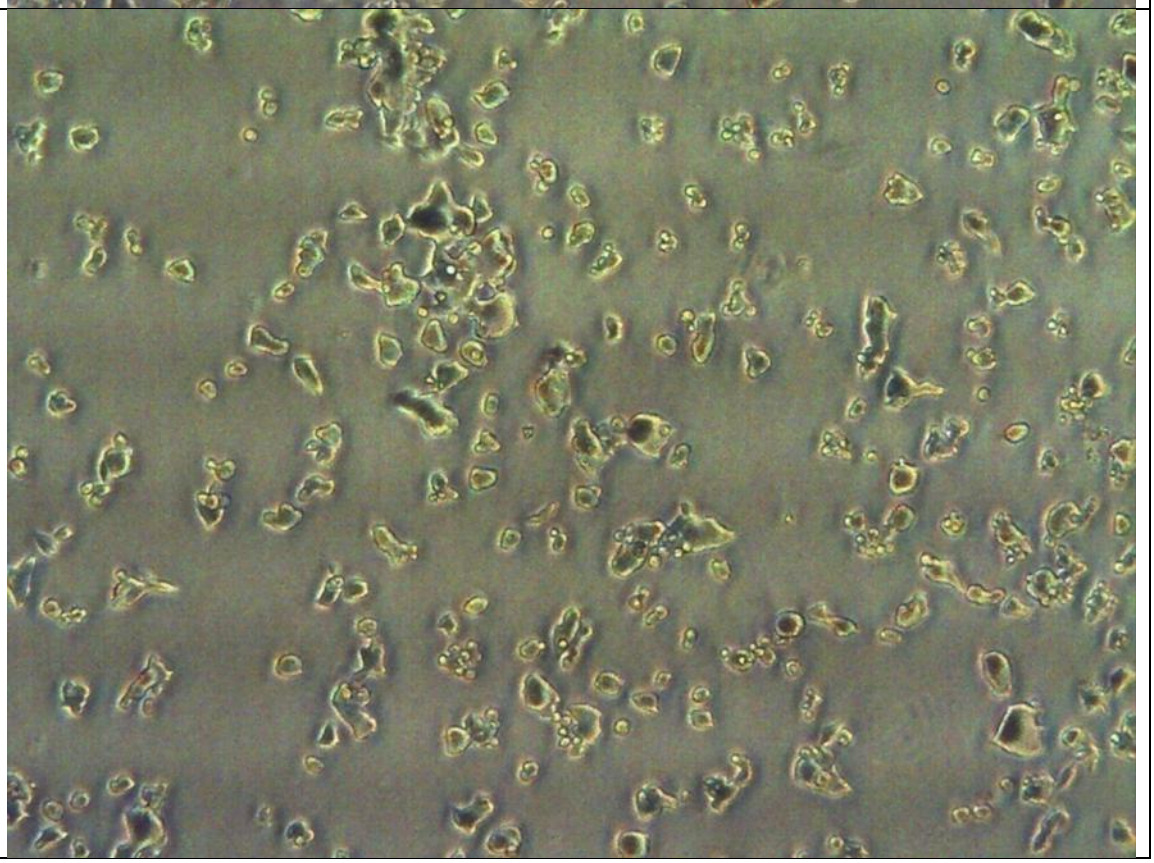
620 24h Bhb 2	
620 24h Bhb 3	

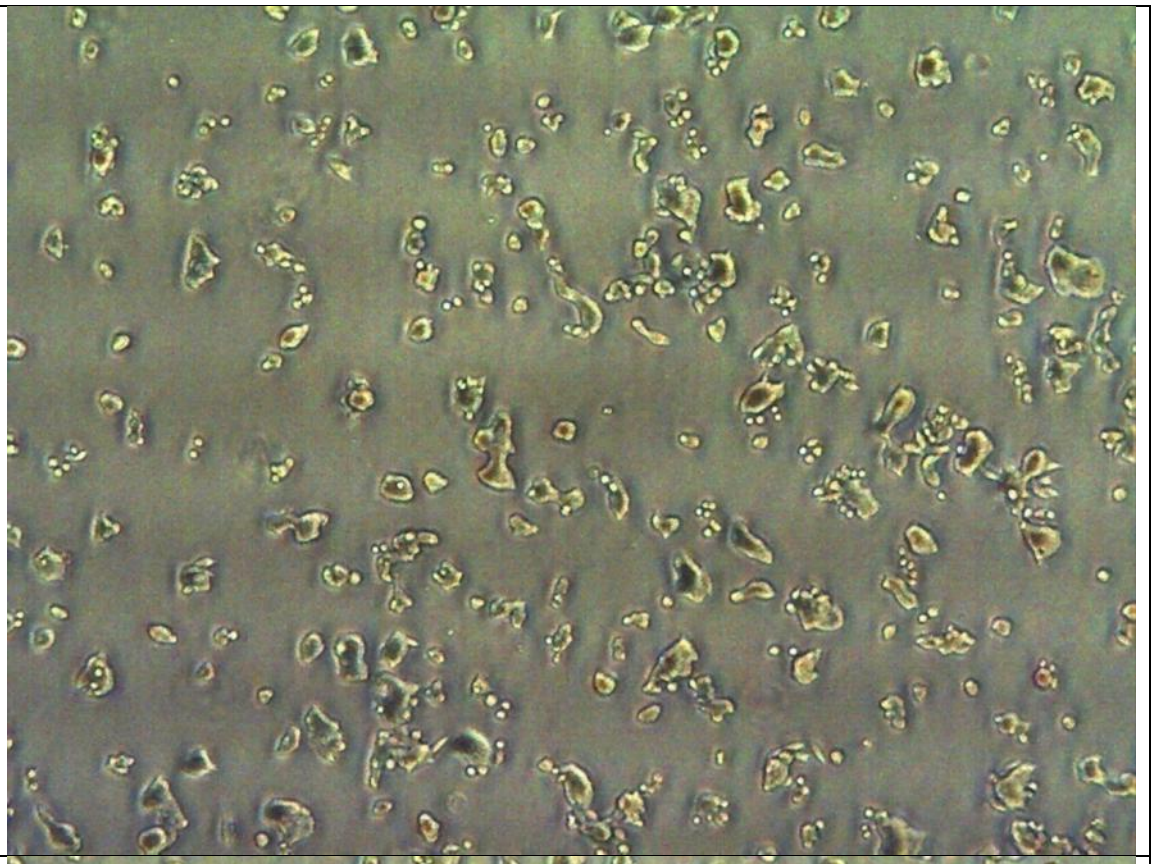
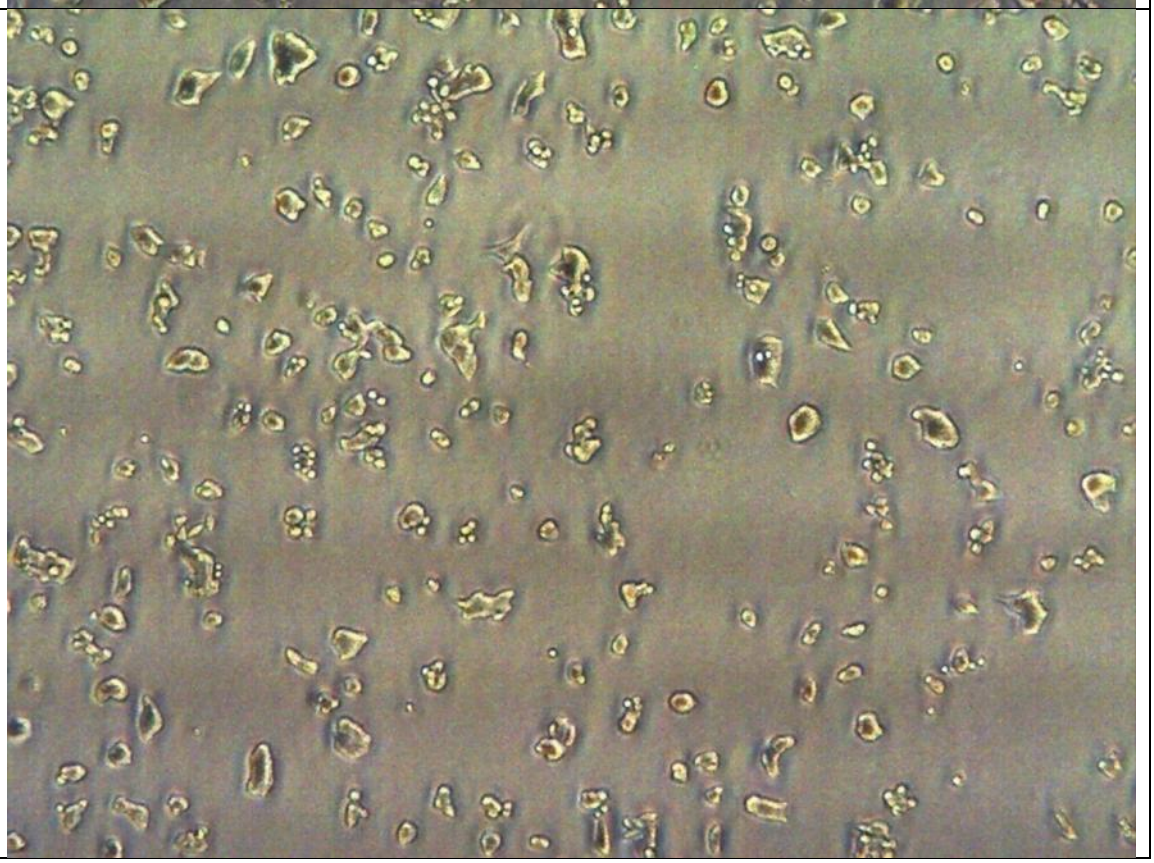
620 48h Cntlr 1	
620 48h Cntlr 2	

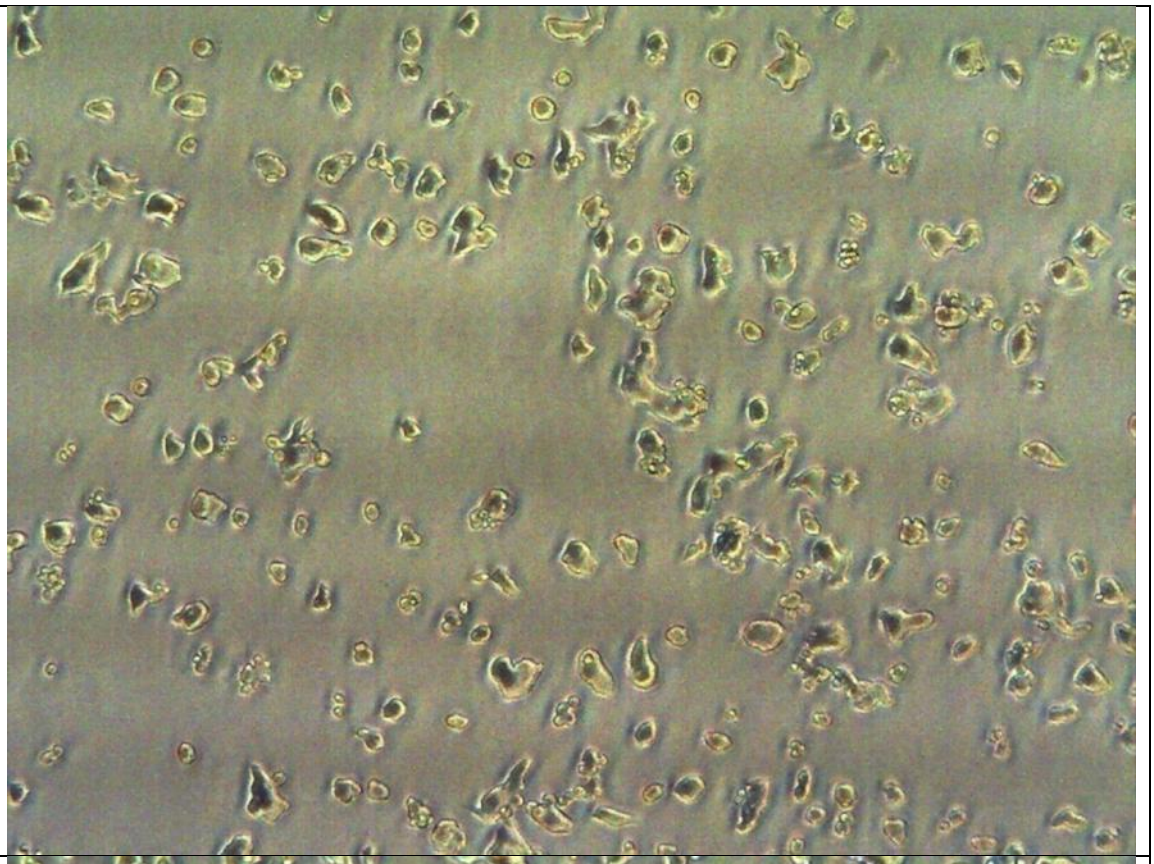
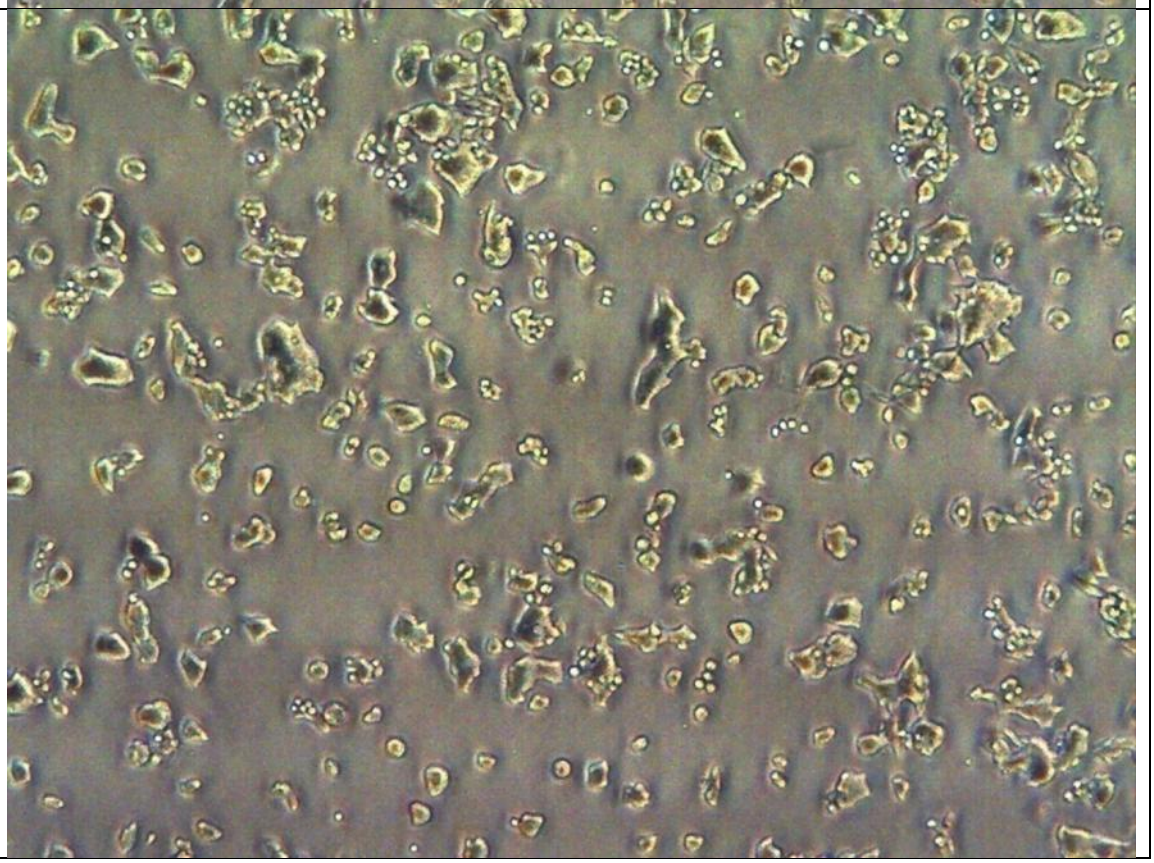
620 48h Cntlr 3	
620 48h Bhb 1	

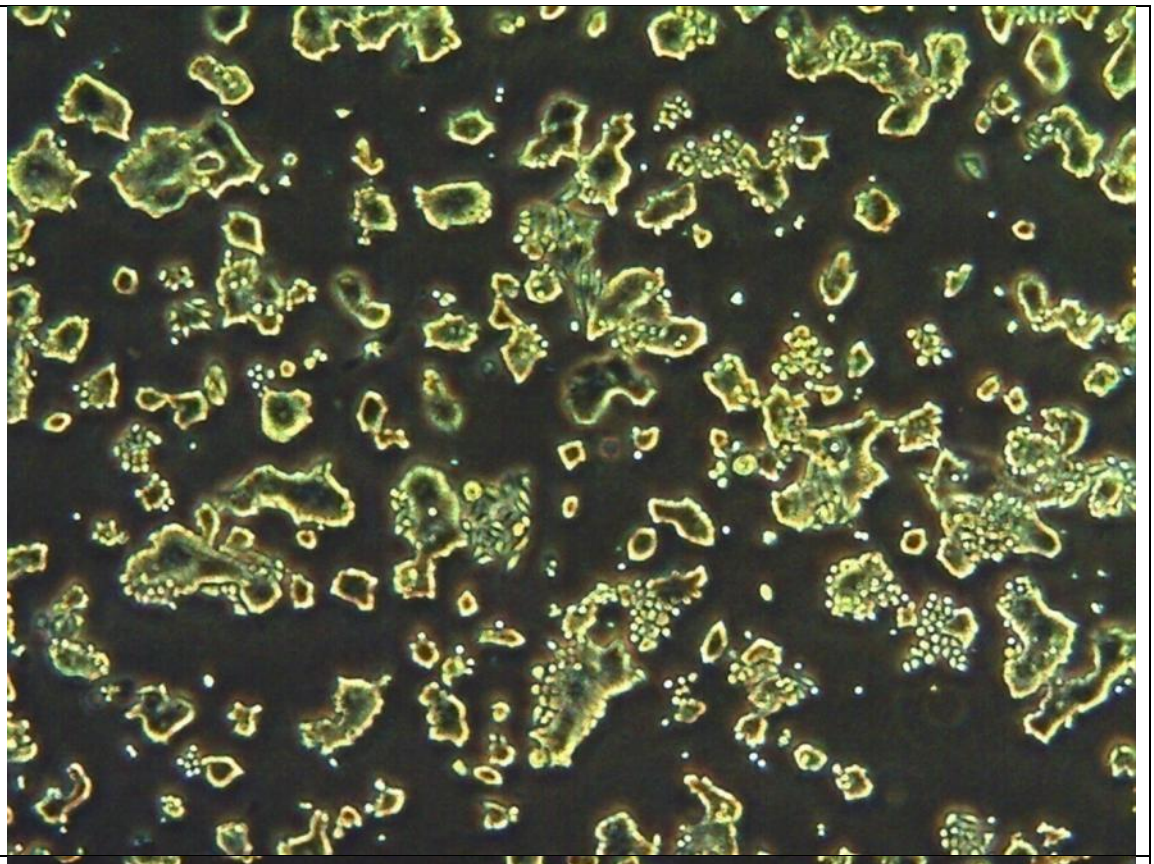
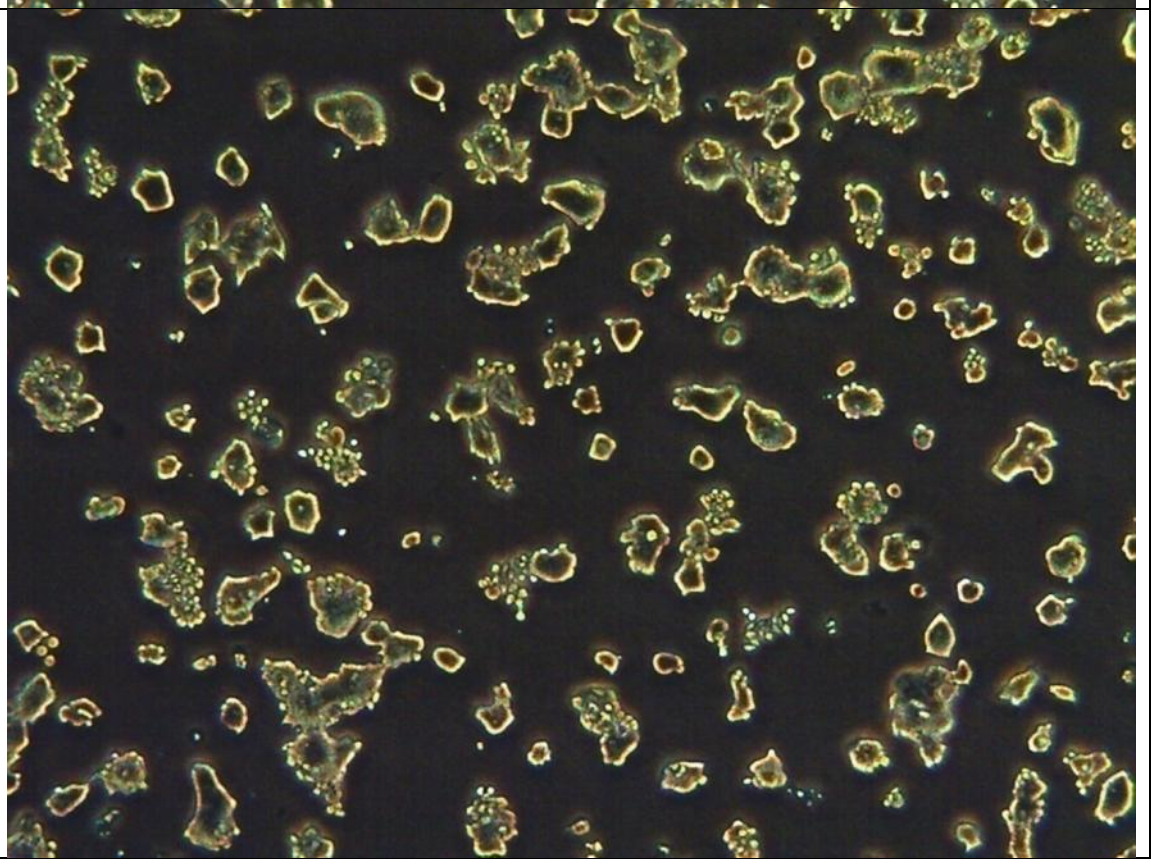
620 48h Bhb 2	
620 48h Bhb 3	

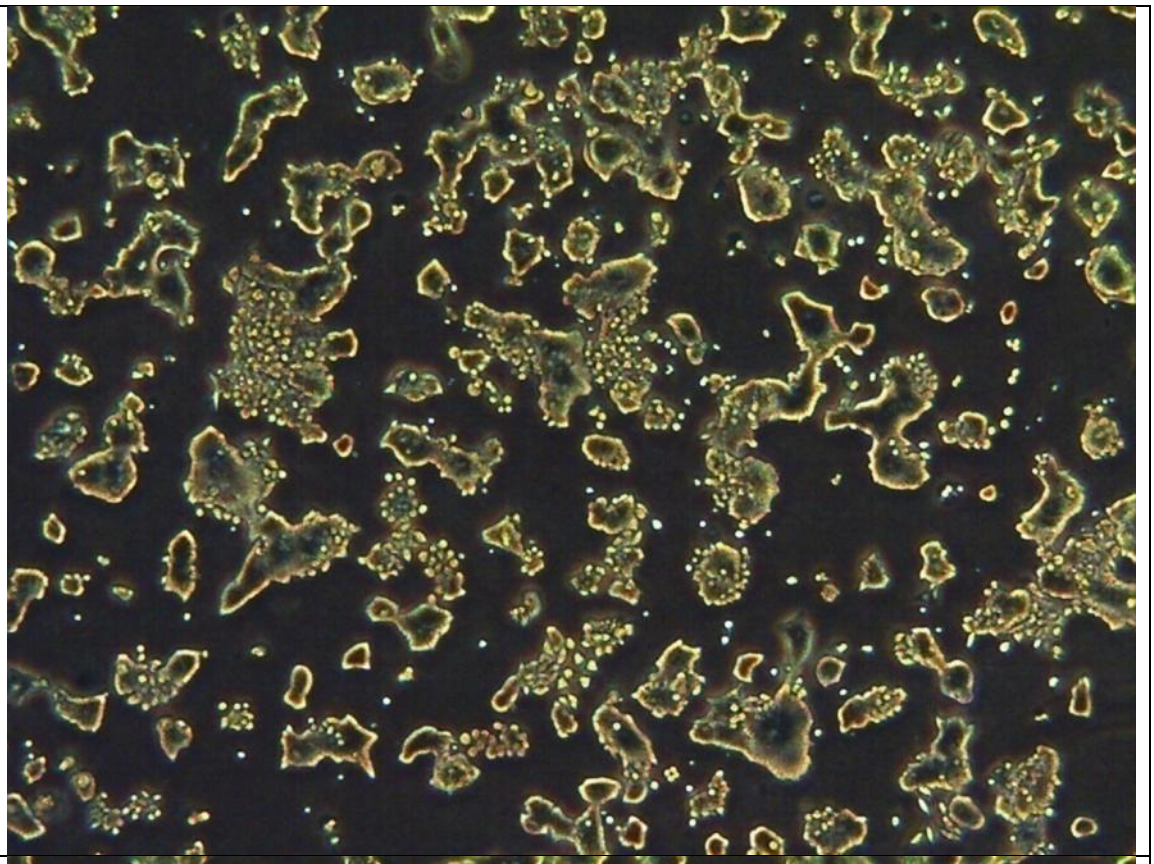
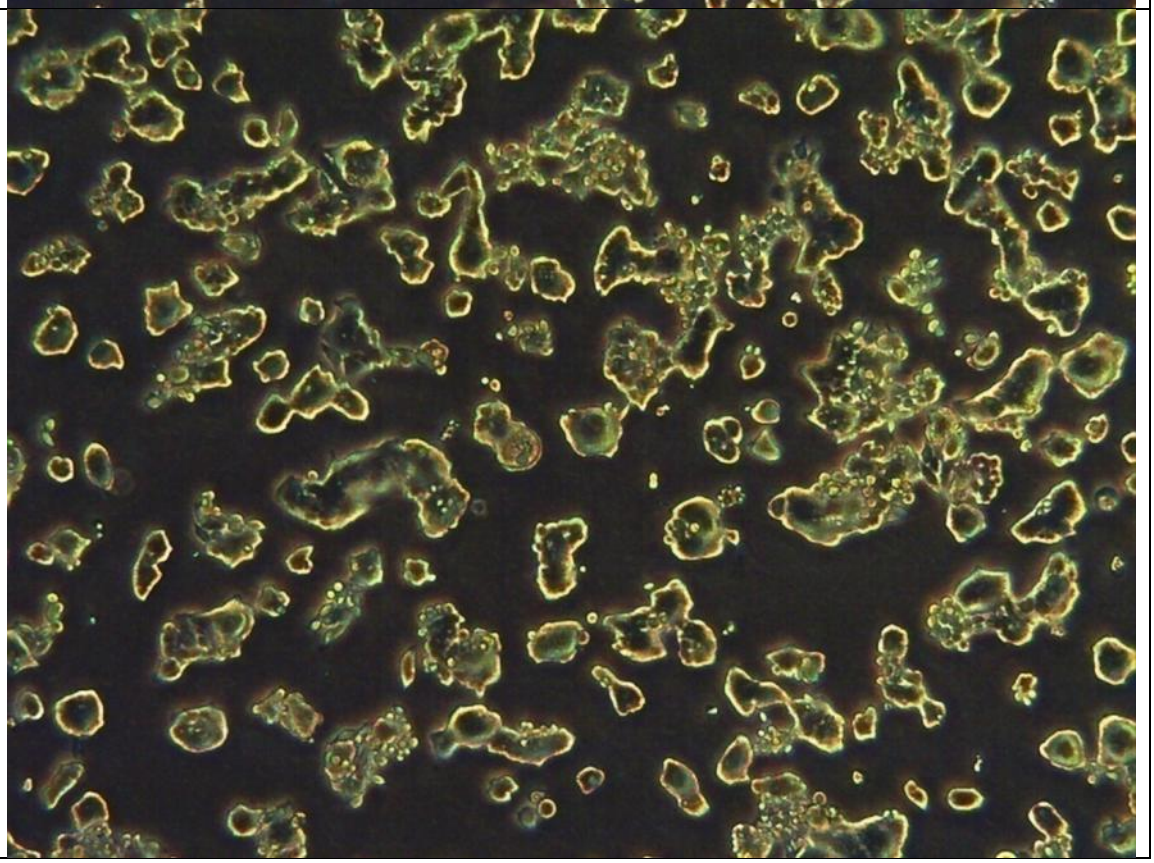
LiM20h1	
LiM20h2	

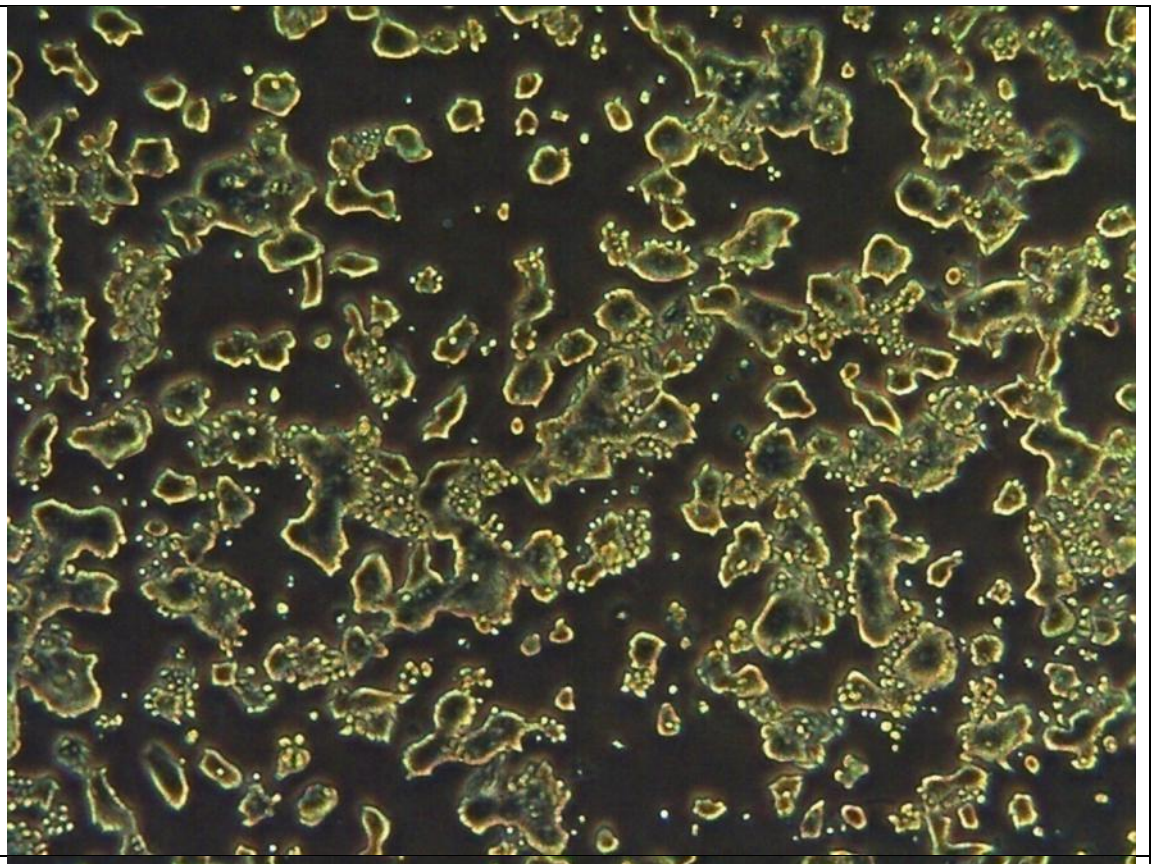
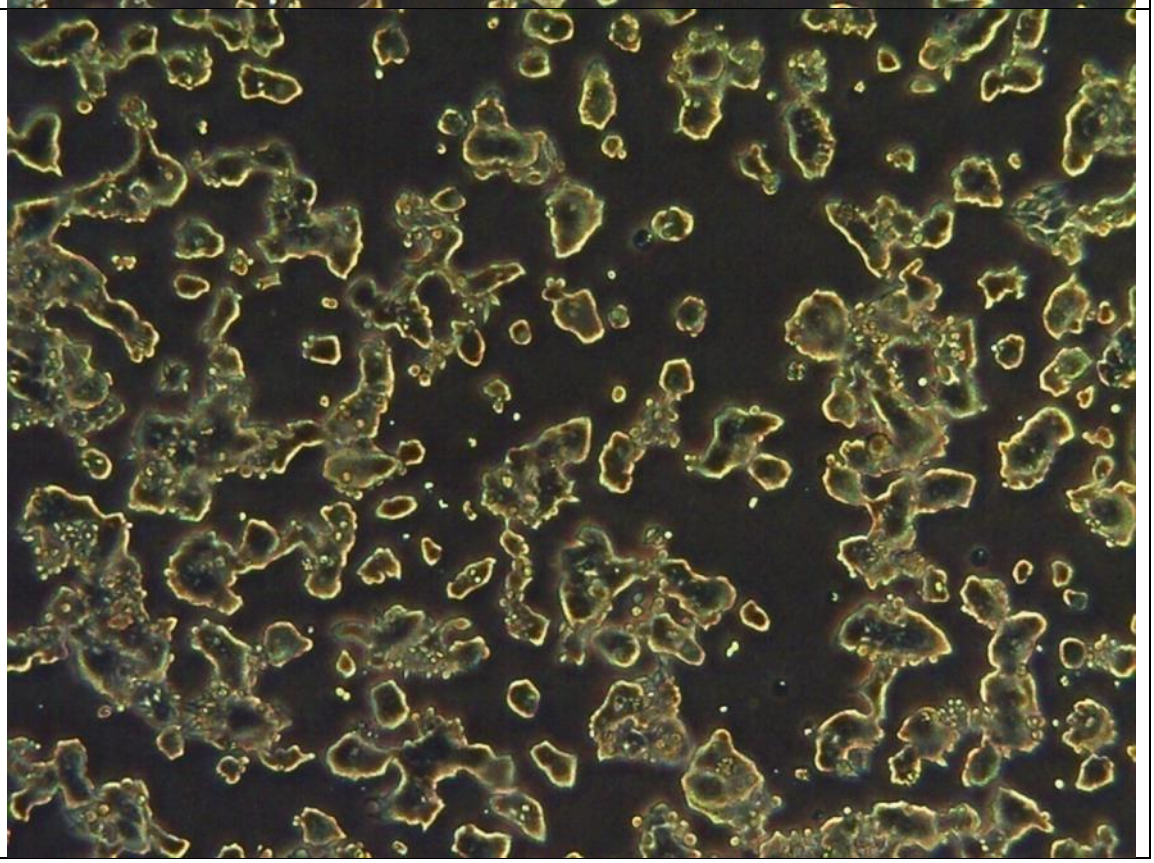
LiM2 24h Cntlr 1	
LiM2 24h Cntlr 2	

LiM2 24h Cntlr 3	
LiM2 24h Bhb 1	

LiM2 24h Bhb 2	
LiM2 24h Bhb 3	

LiM2 48h Cntlr 1	
LiM2 48h Cntlr 2	

LiM2 48h Cntlr 3	
LiM2 48h Bhb 1	

LiM2 48h Bhb 2	
LiM2 48h Bhb 3	

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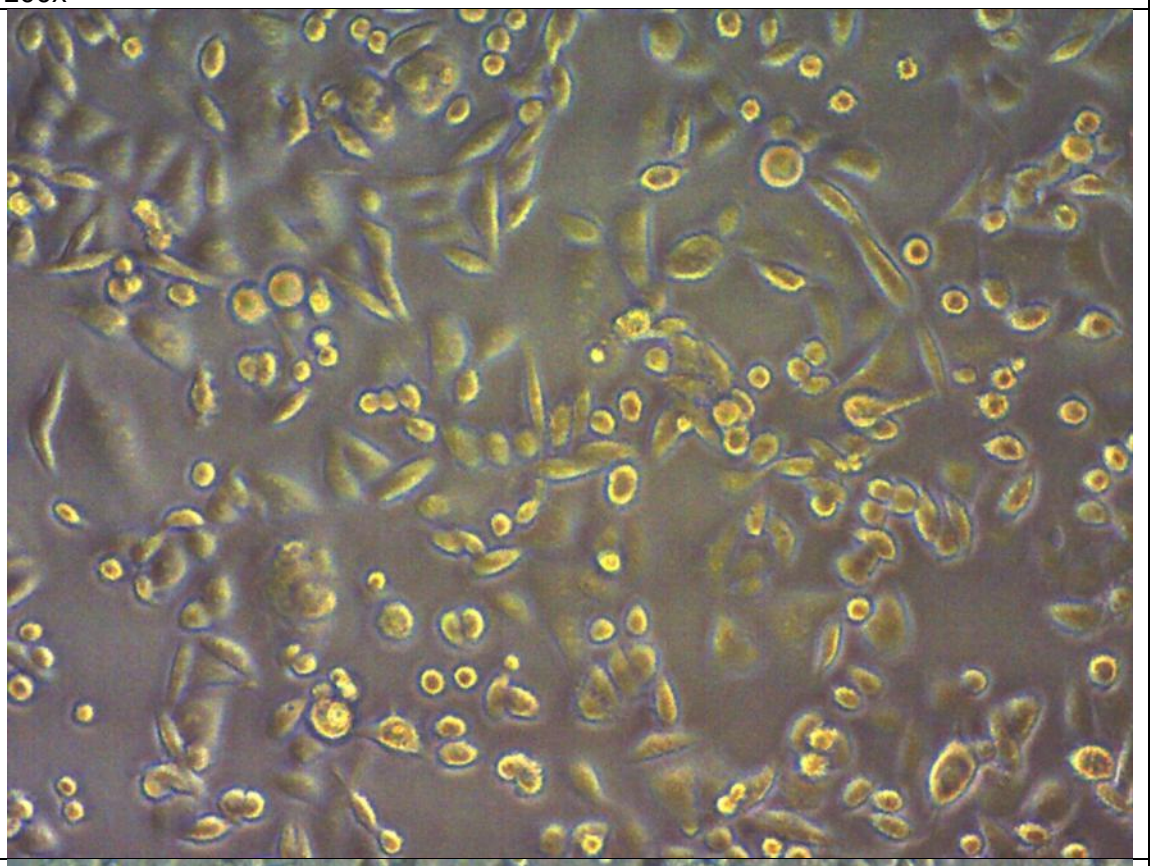
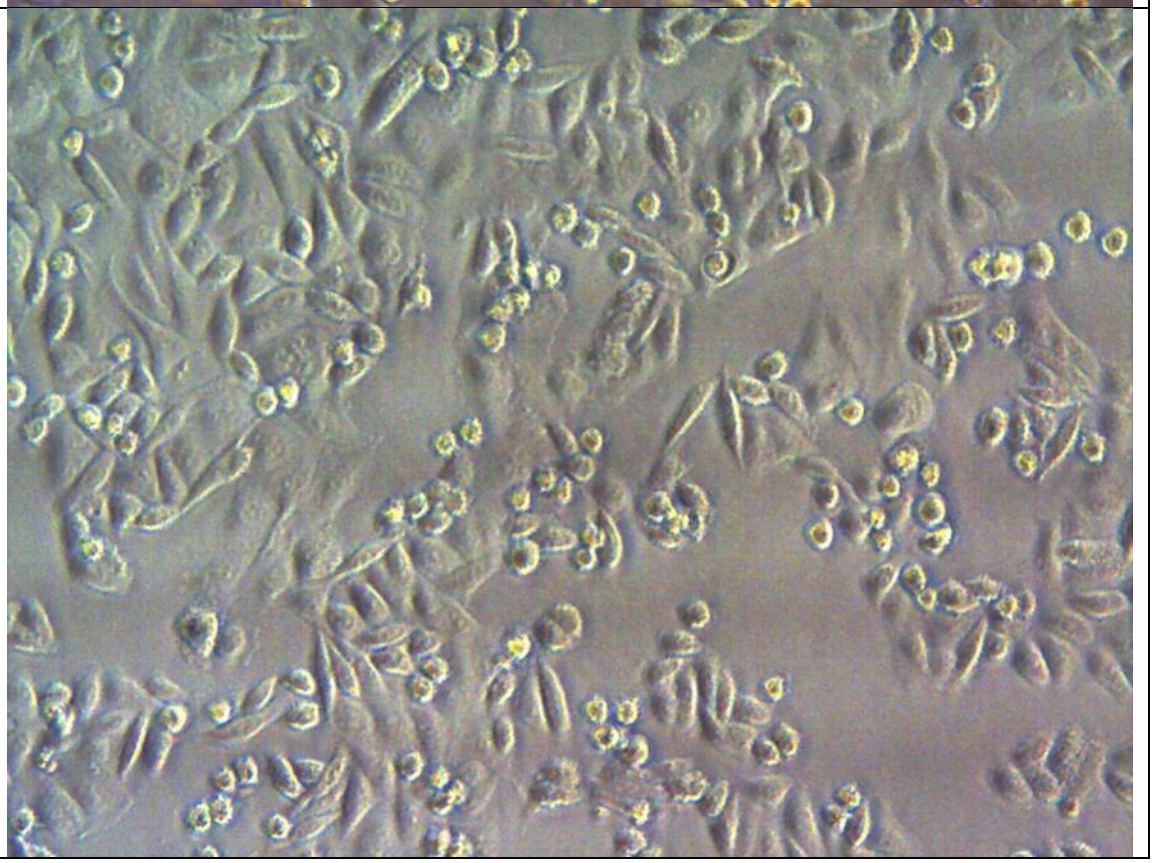
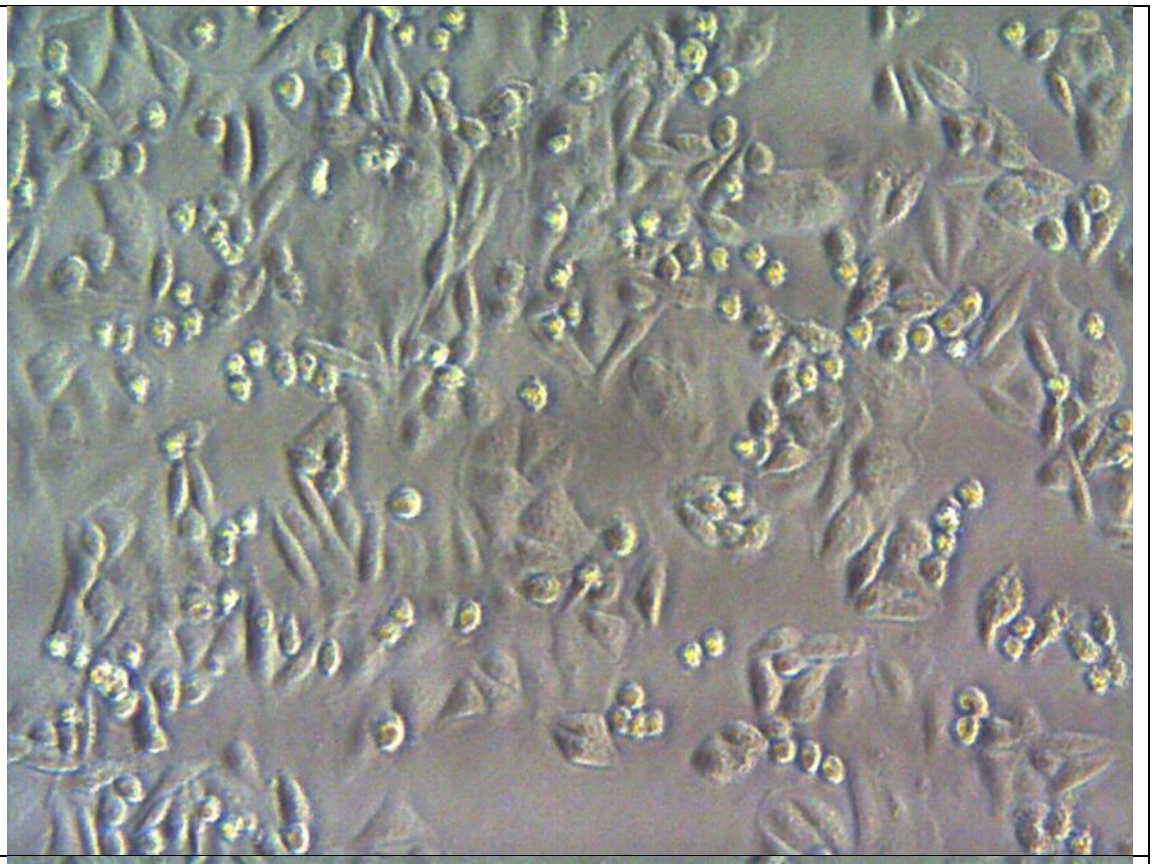
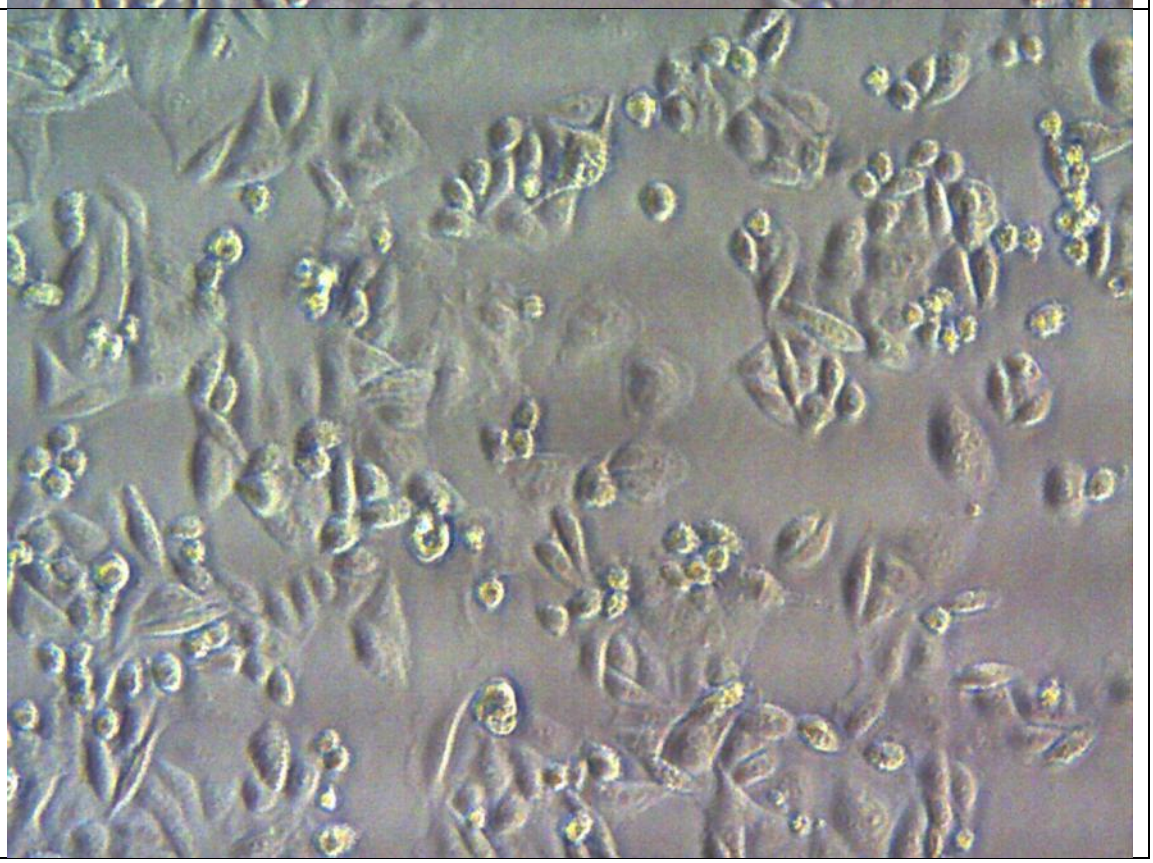
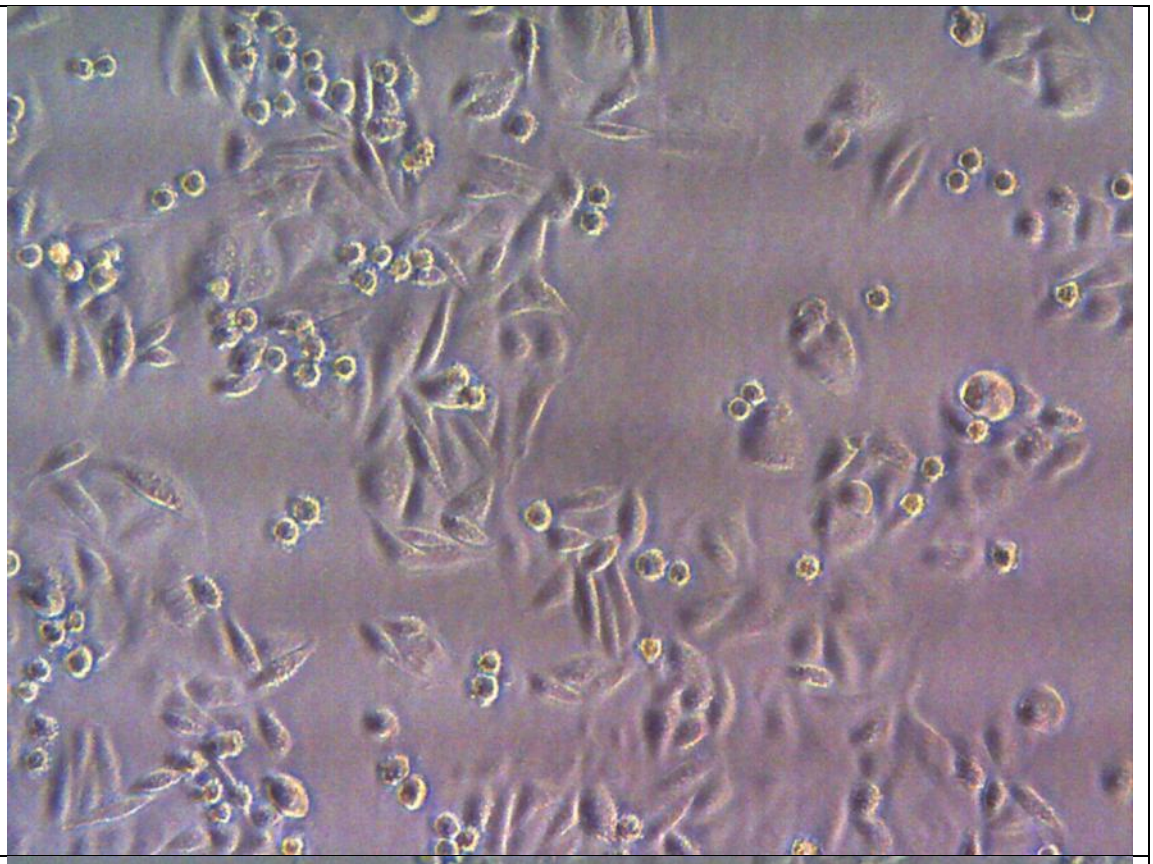
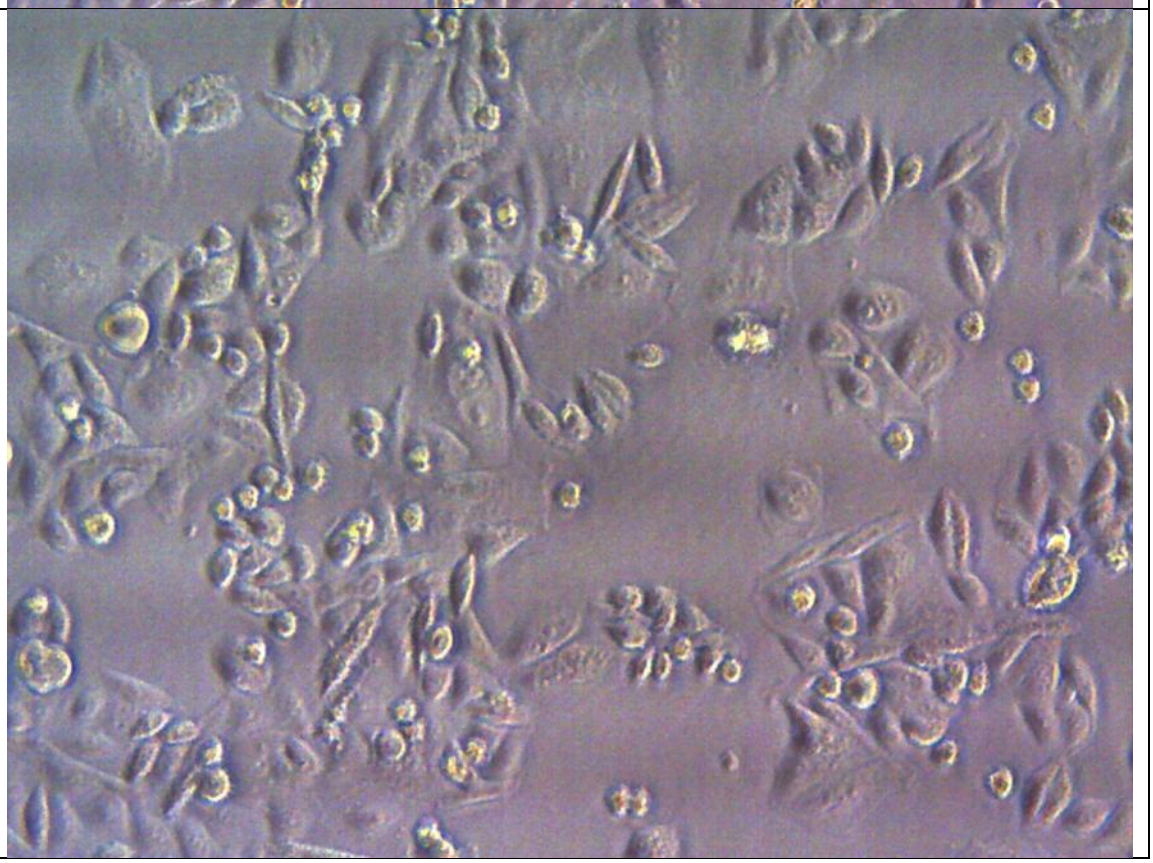
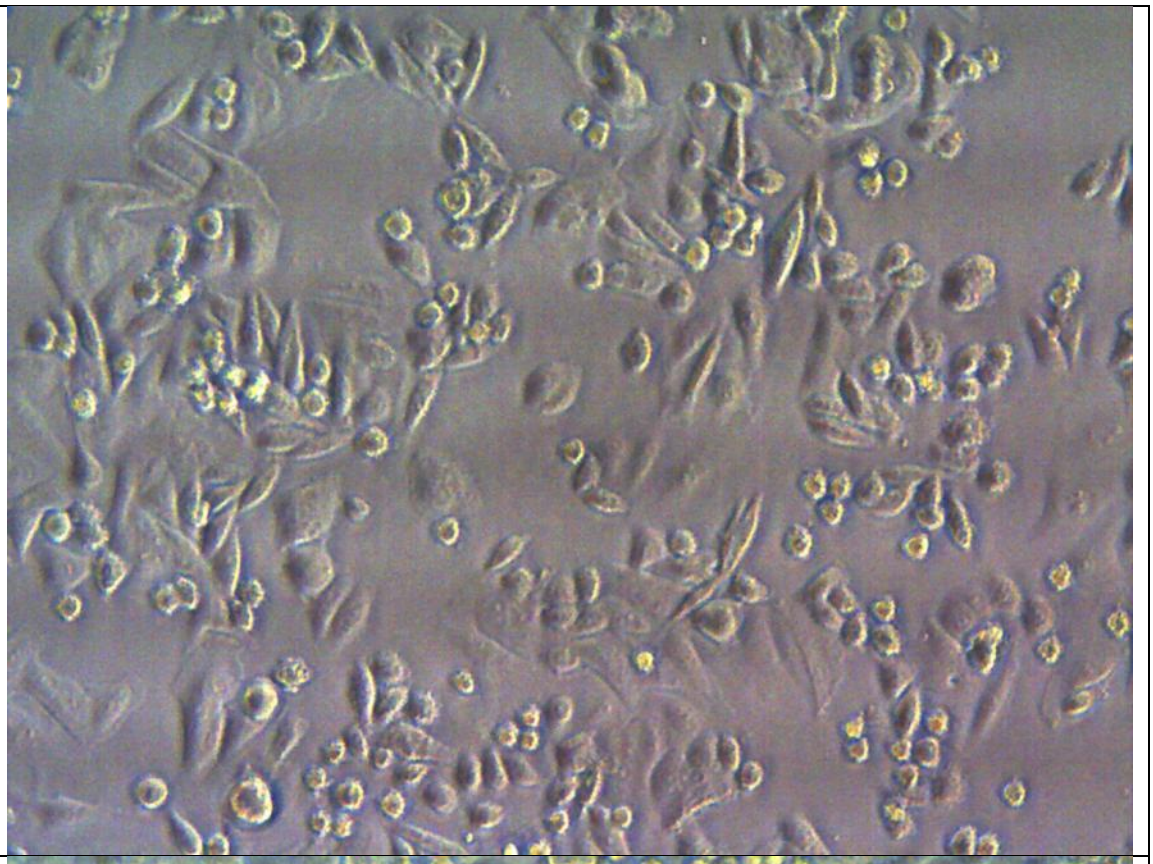
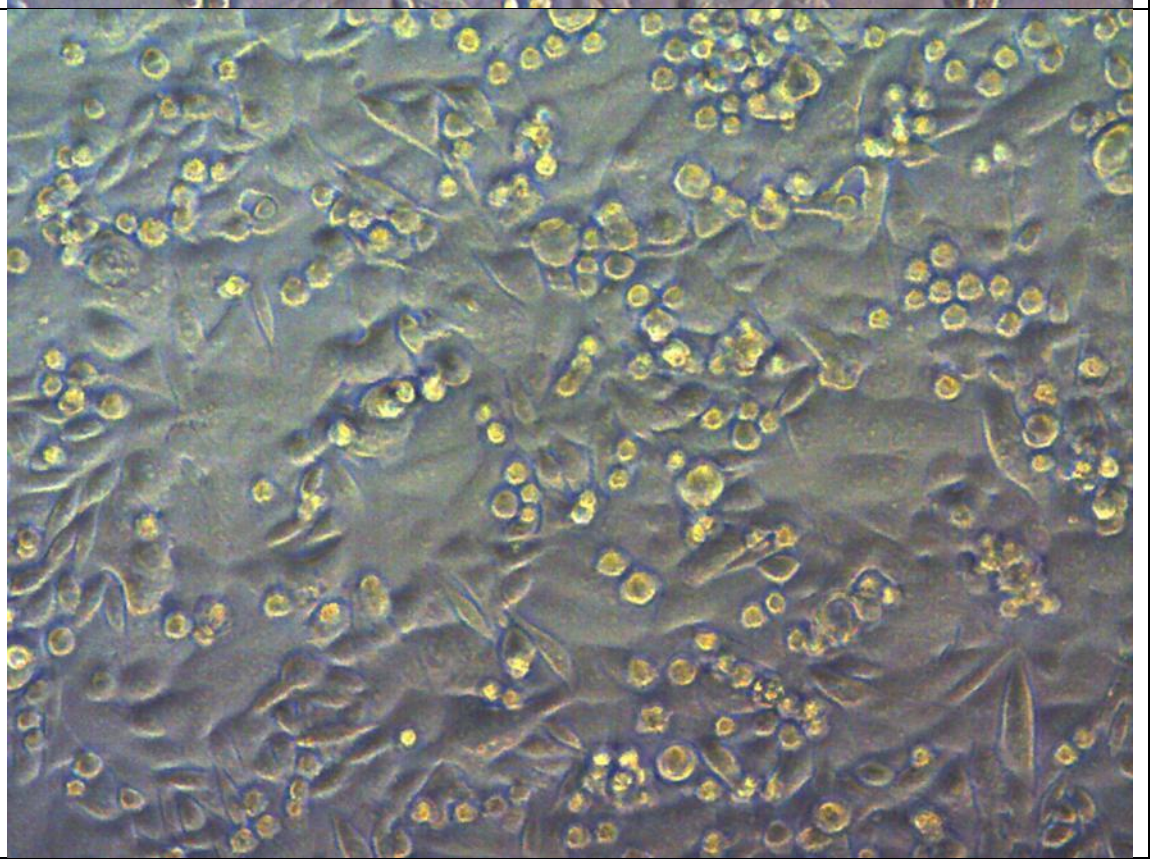
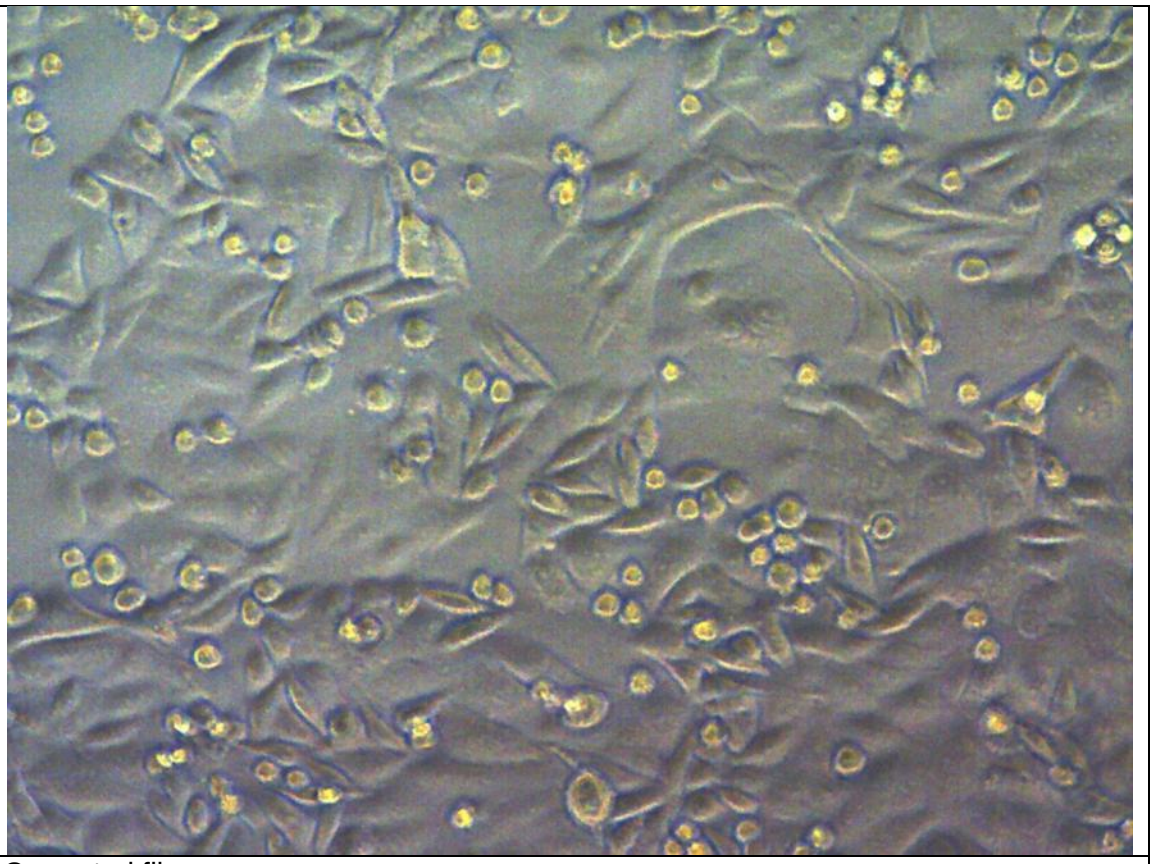
480 0h 1	
480 24h Cntlr 1	

480 24h Cntlr 2	
480 24h Cntlr 3	

480 24h Bhb 1	
480 24h Bhb 2	

480 24h Bhb 3	
480 48h Cntlr 1	

480 48h Cntlr 2	
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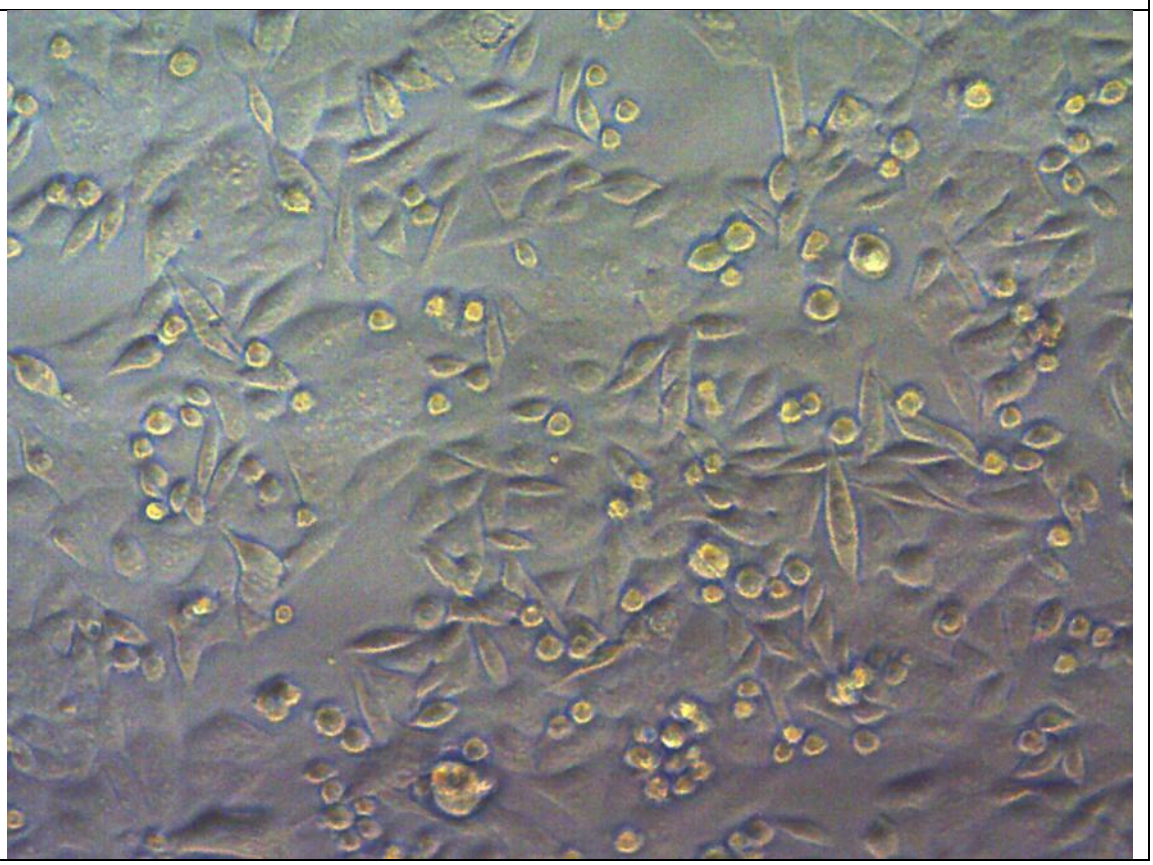
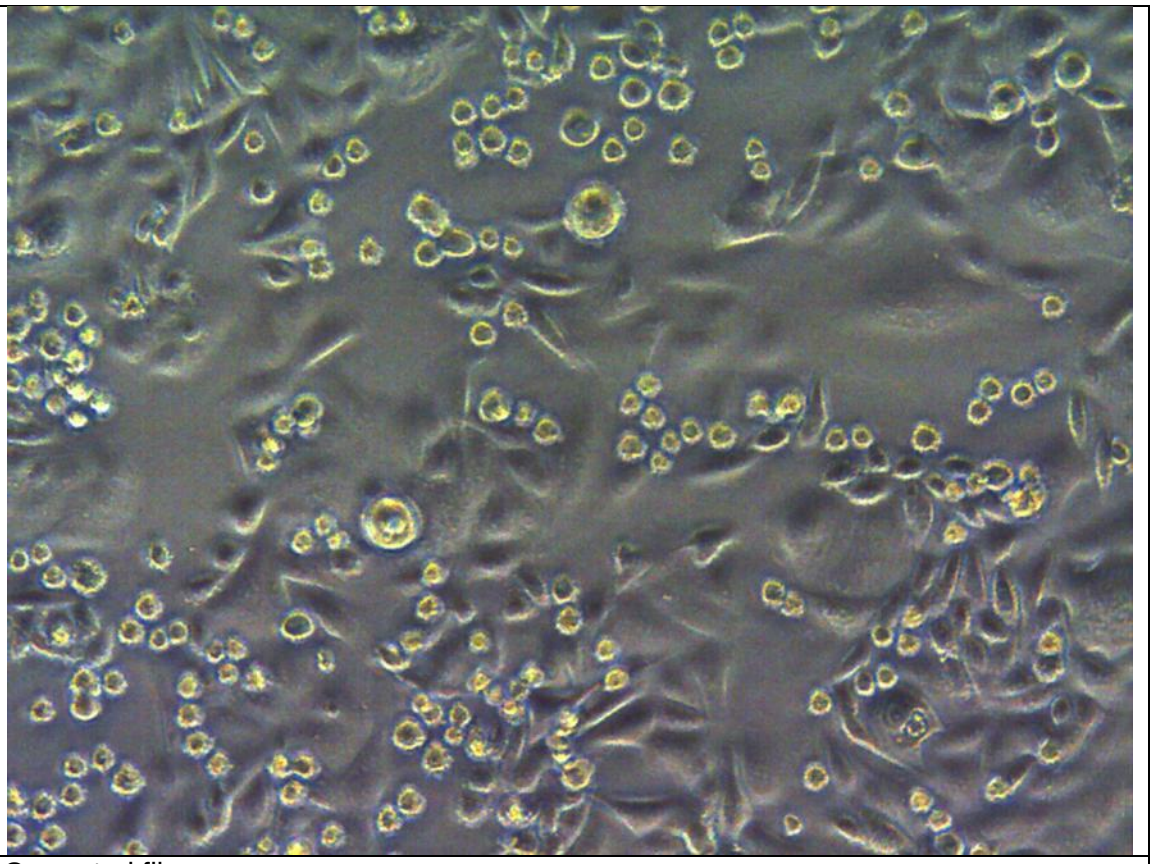
480 48h Bhb 1	

480 48h Bhb 2	
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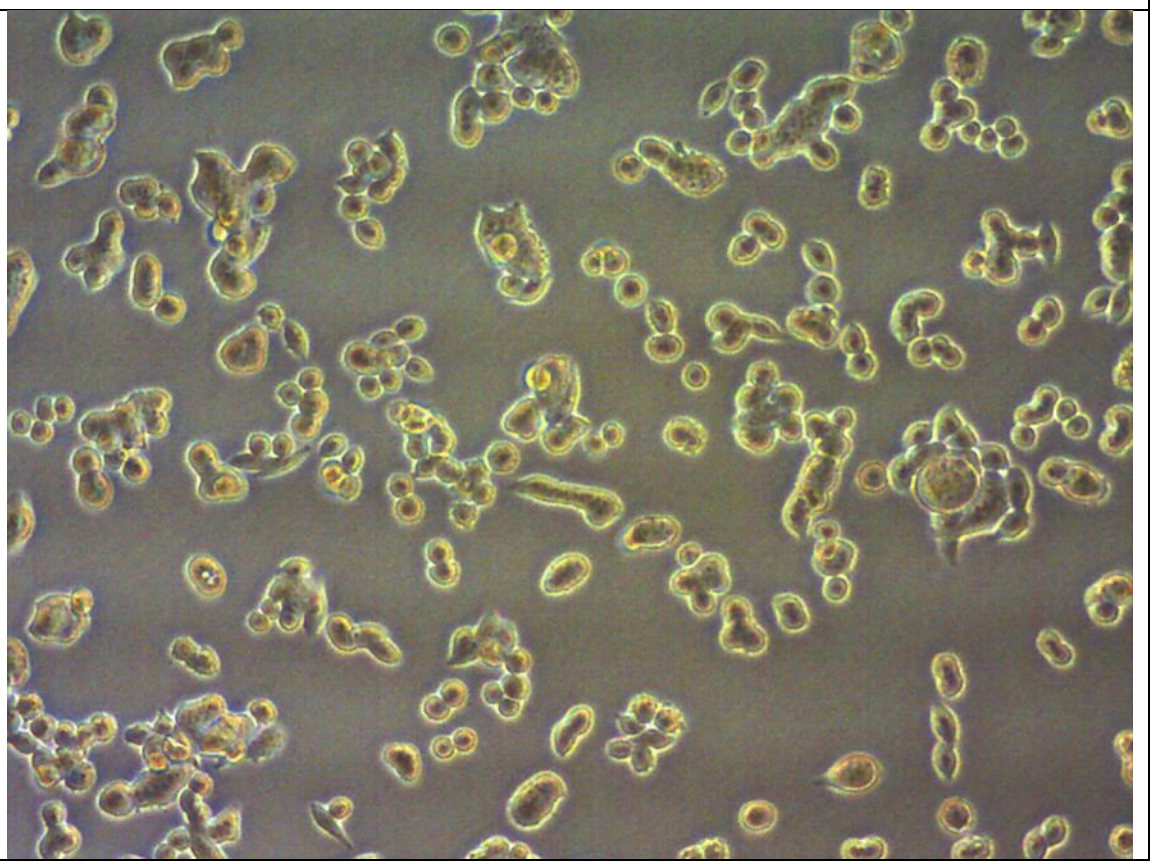
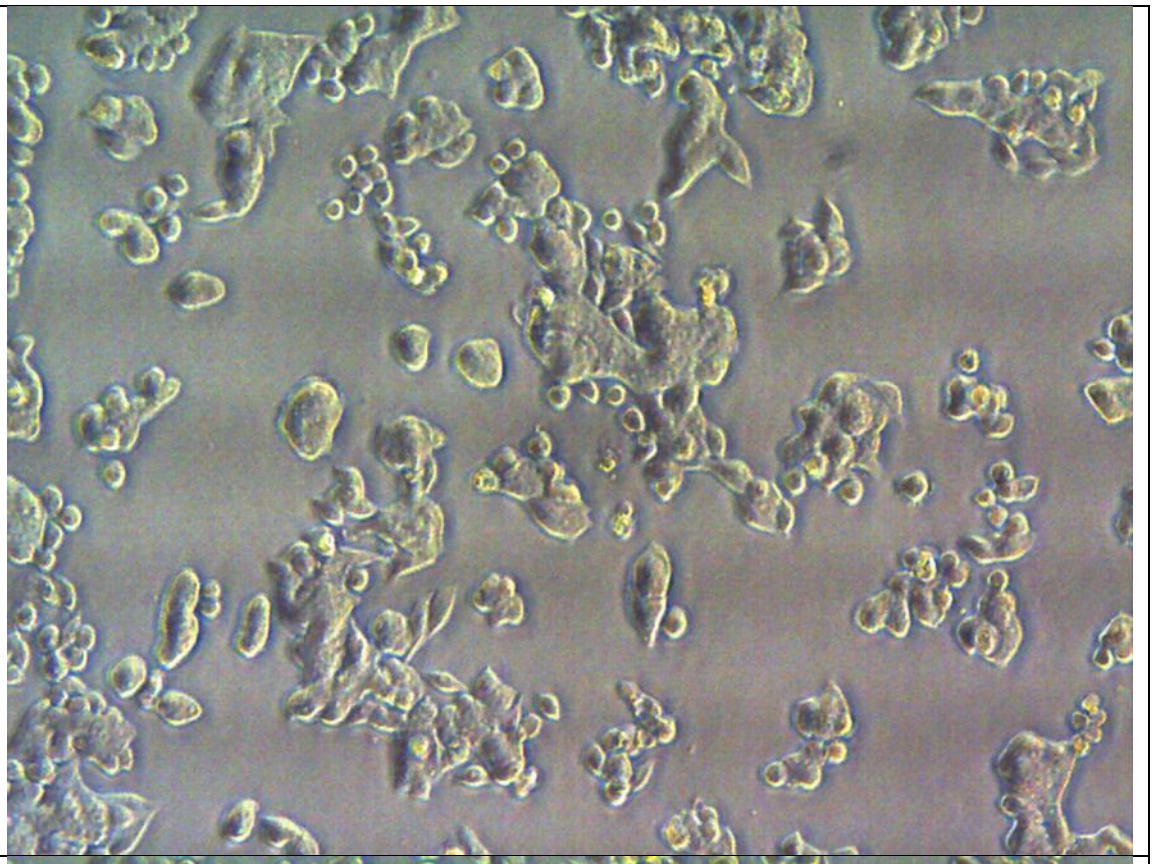
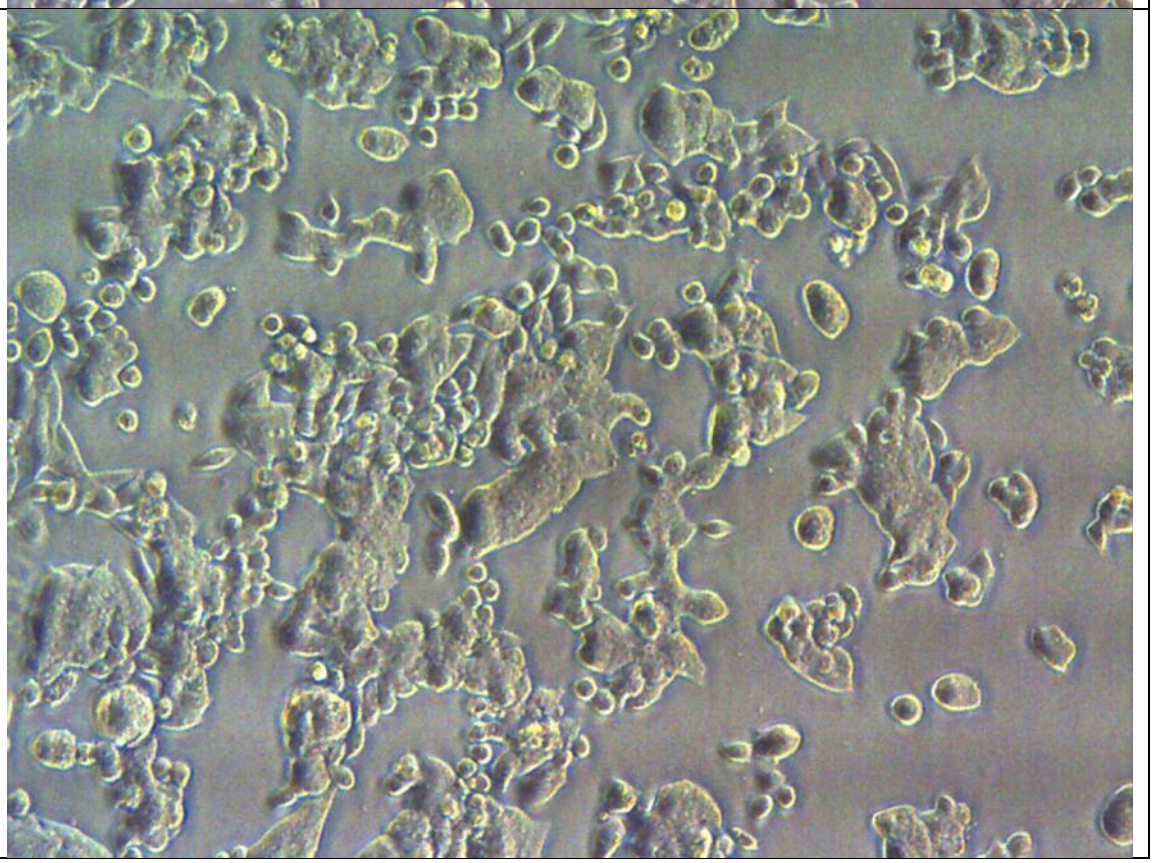
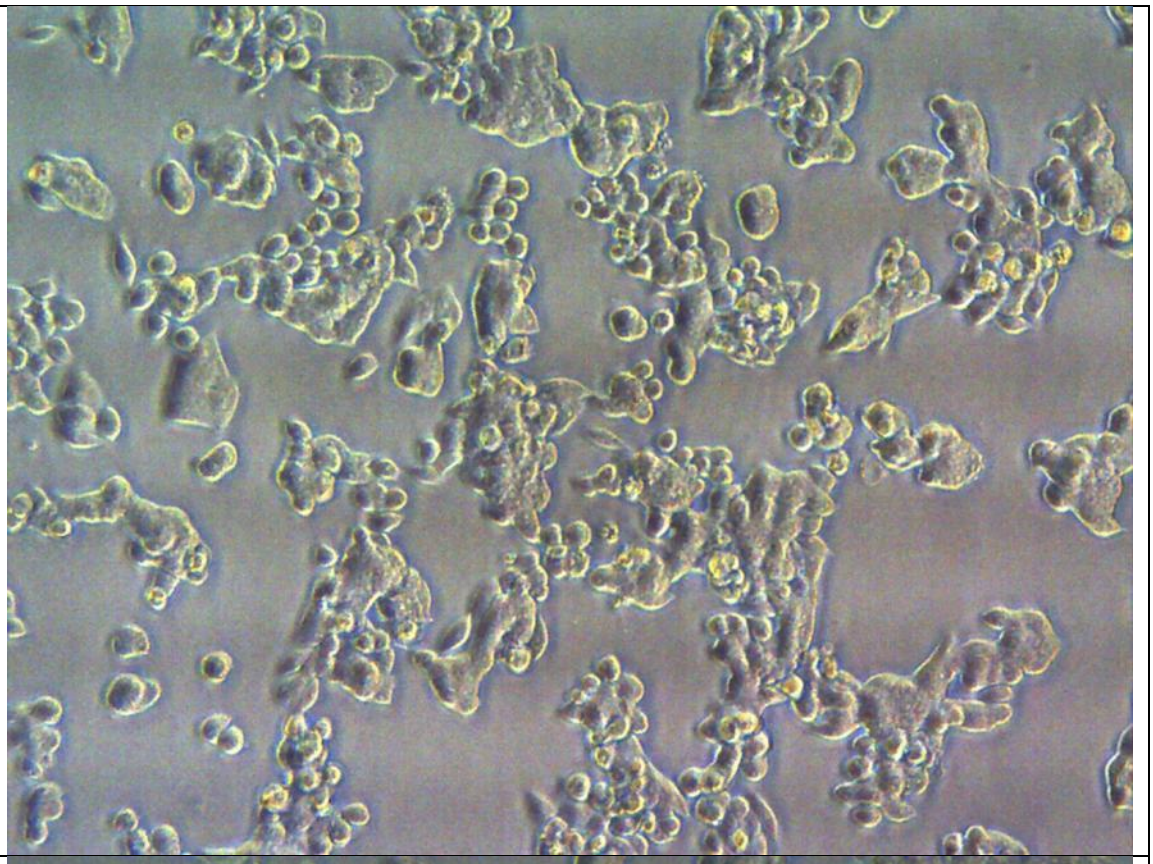
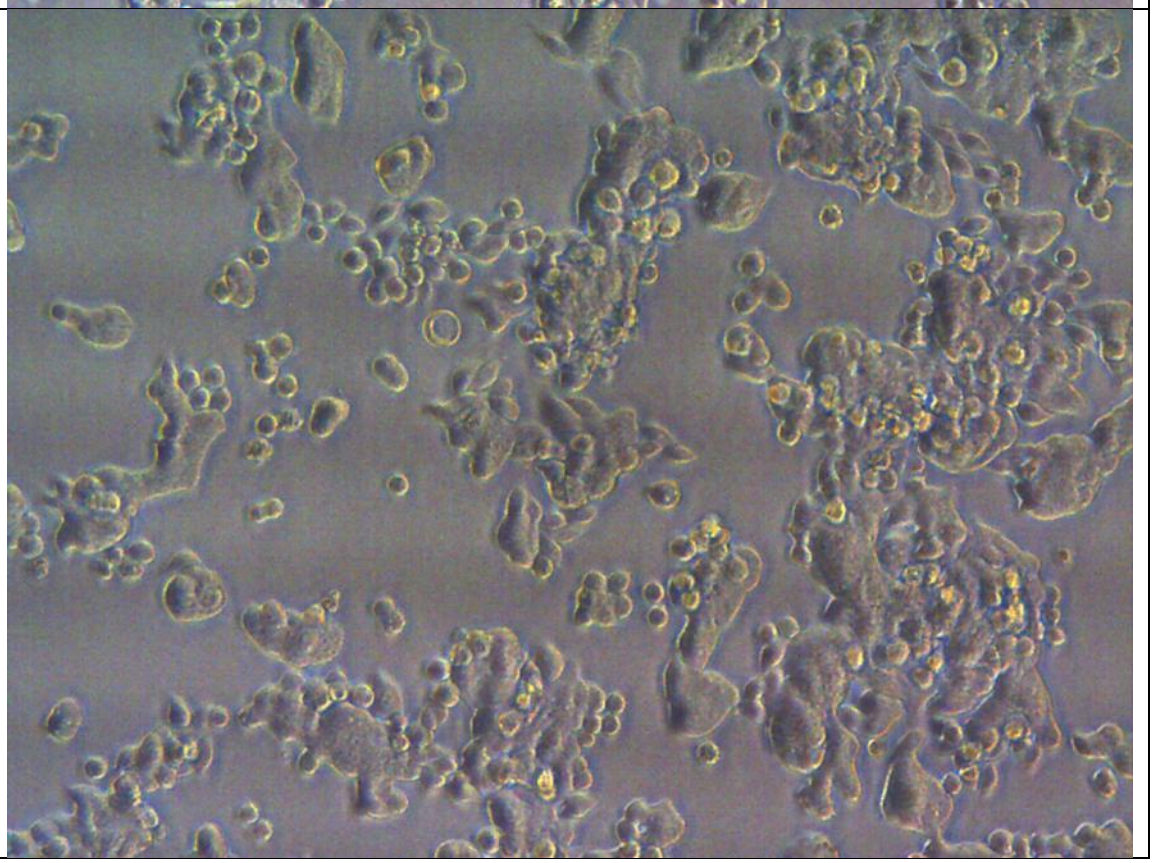
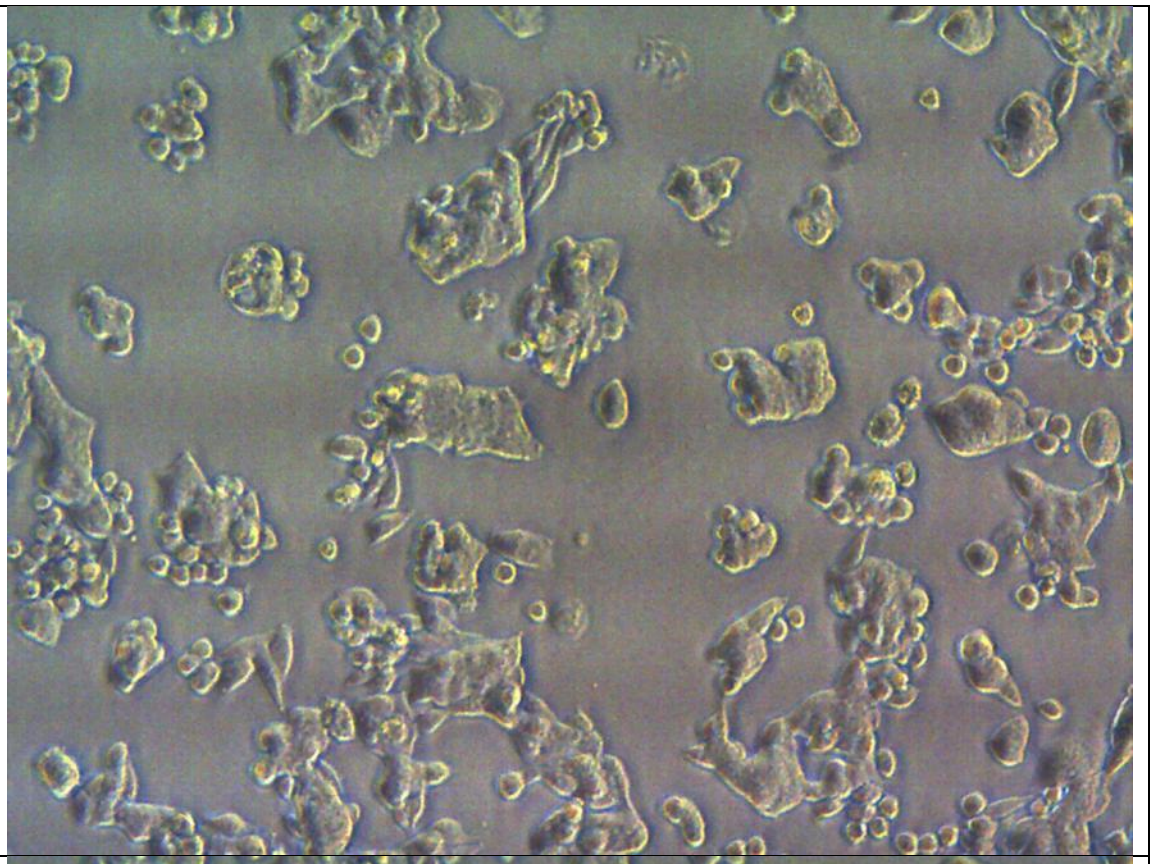
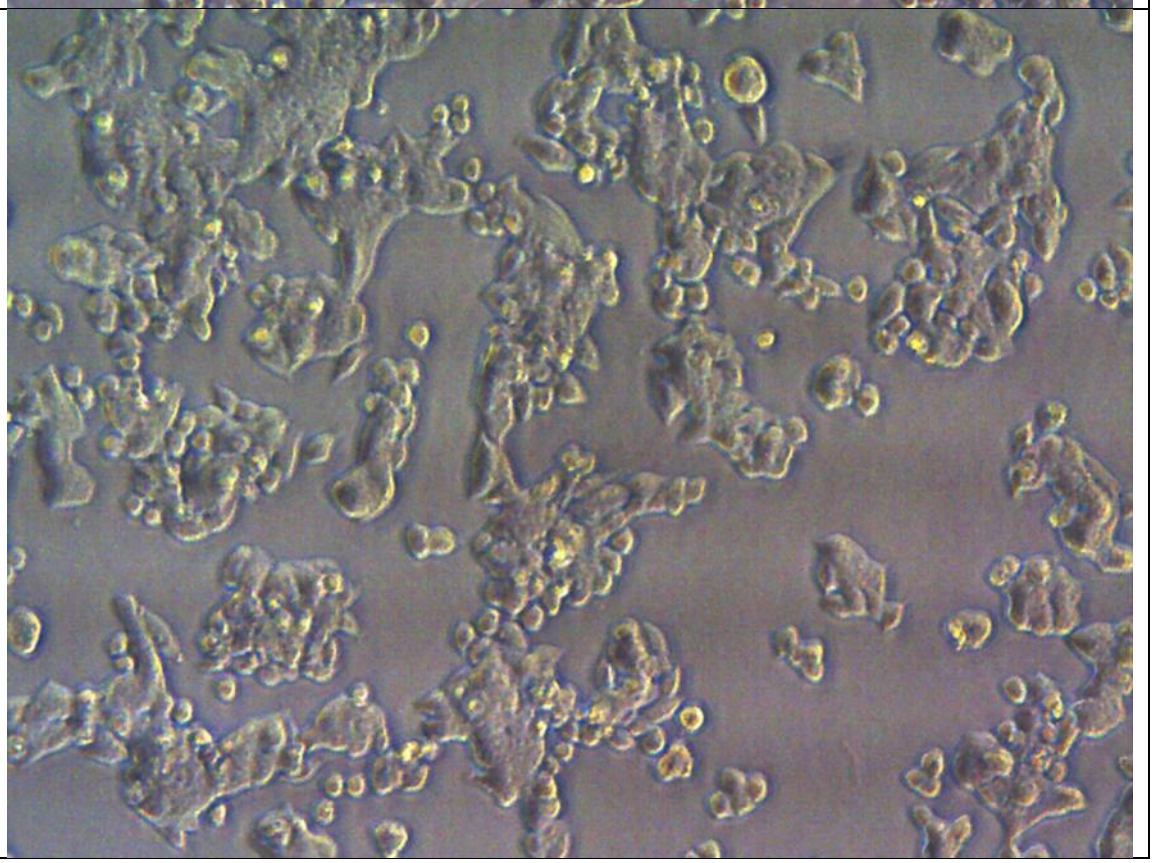
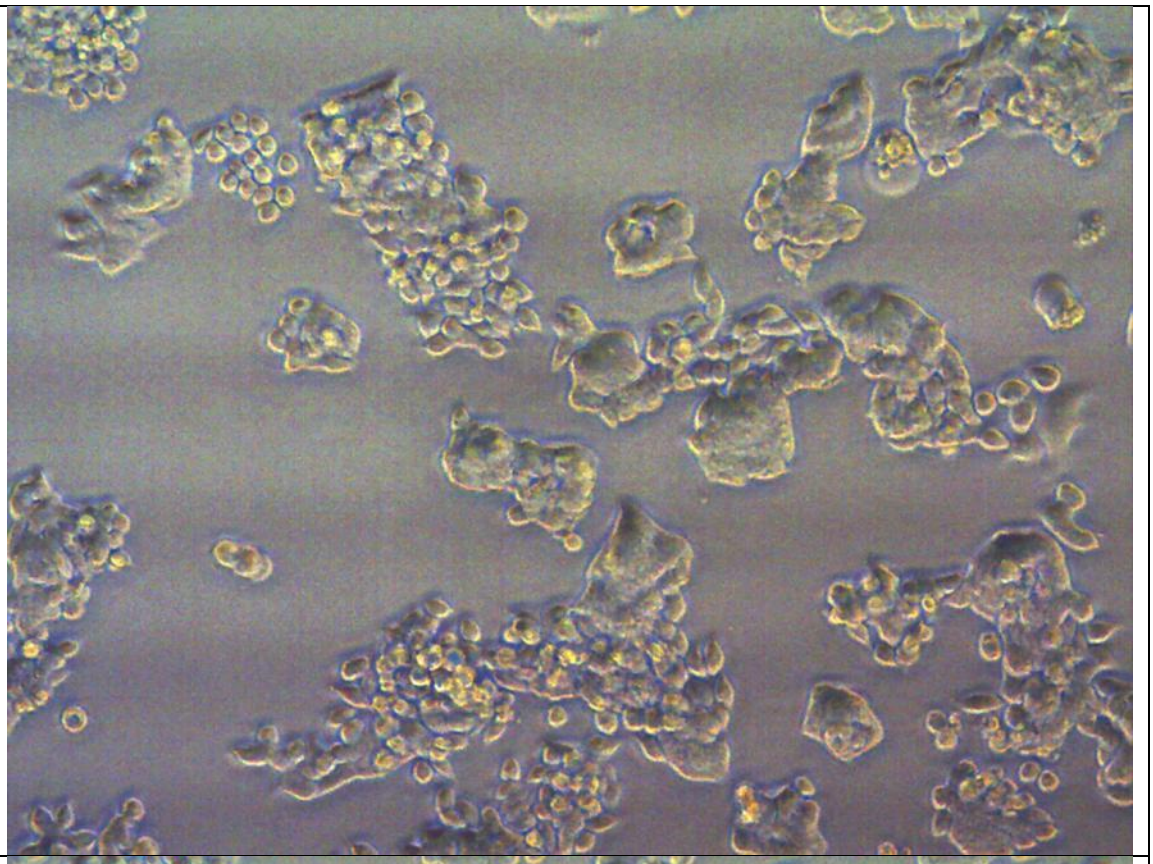
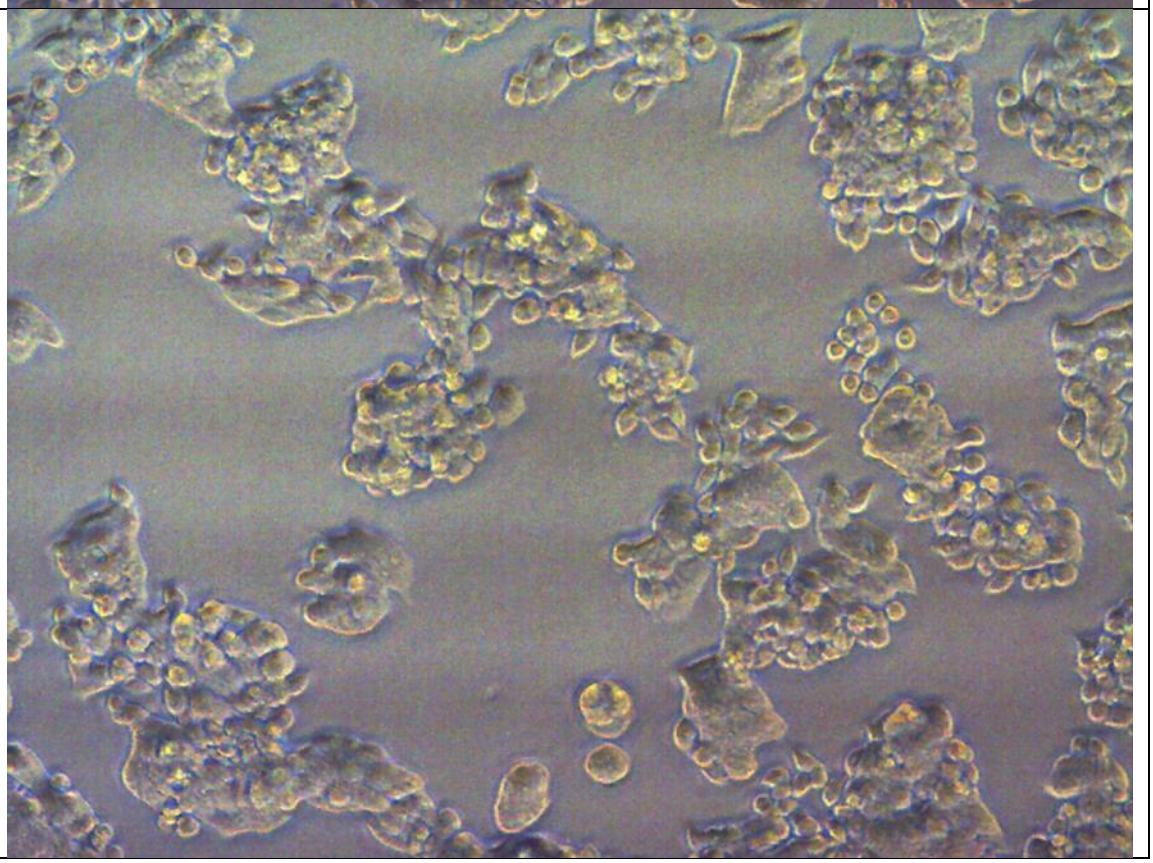
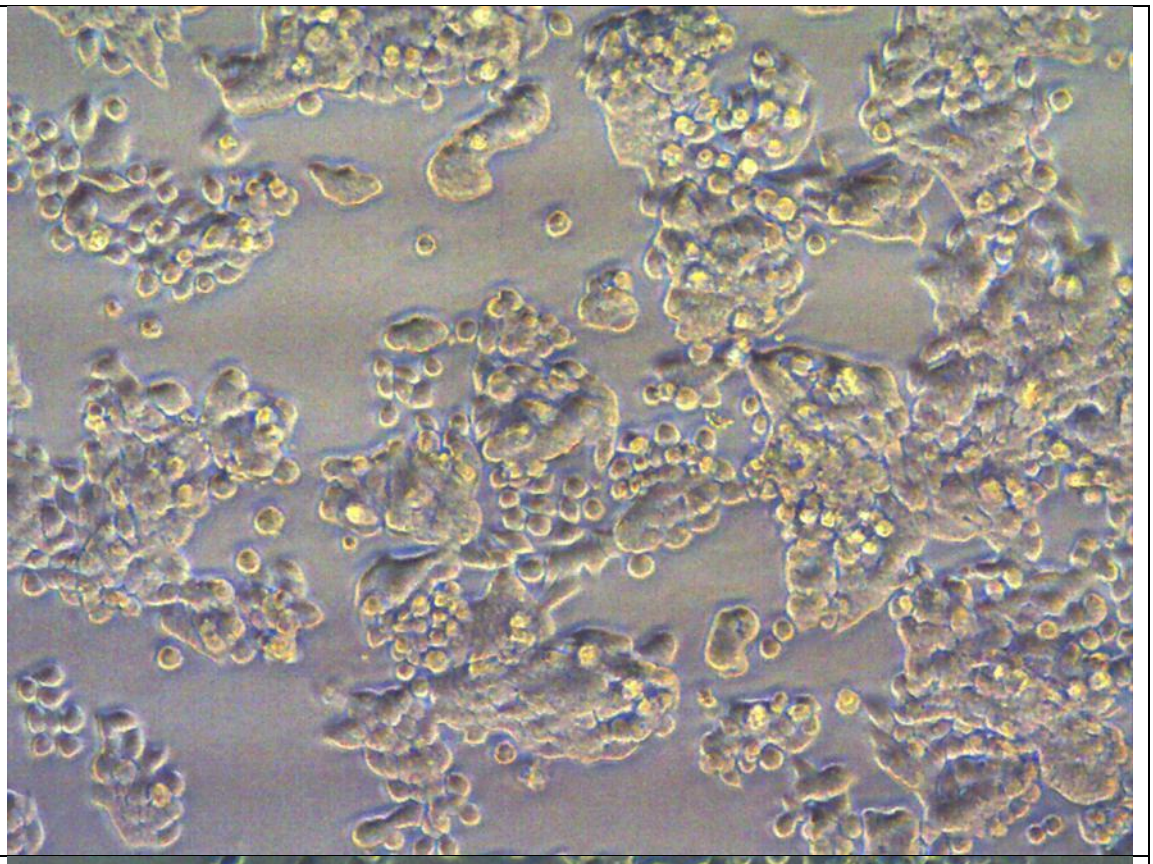
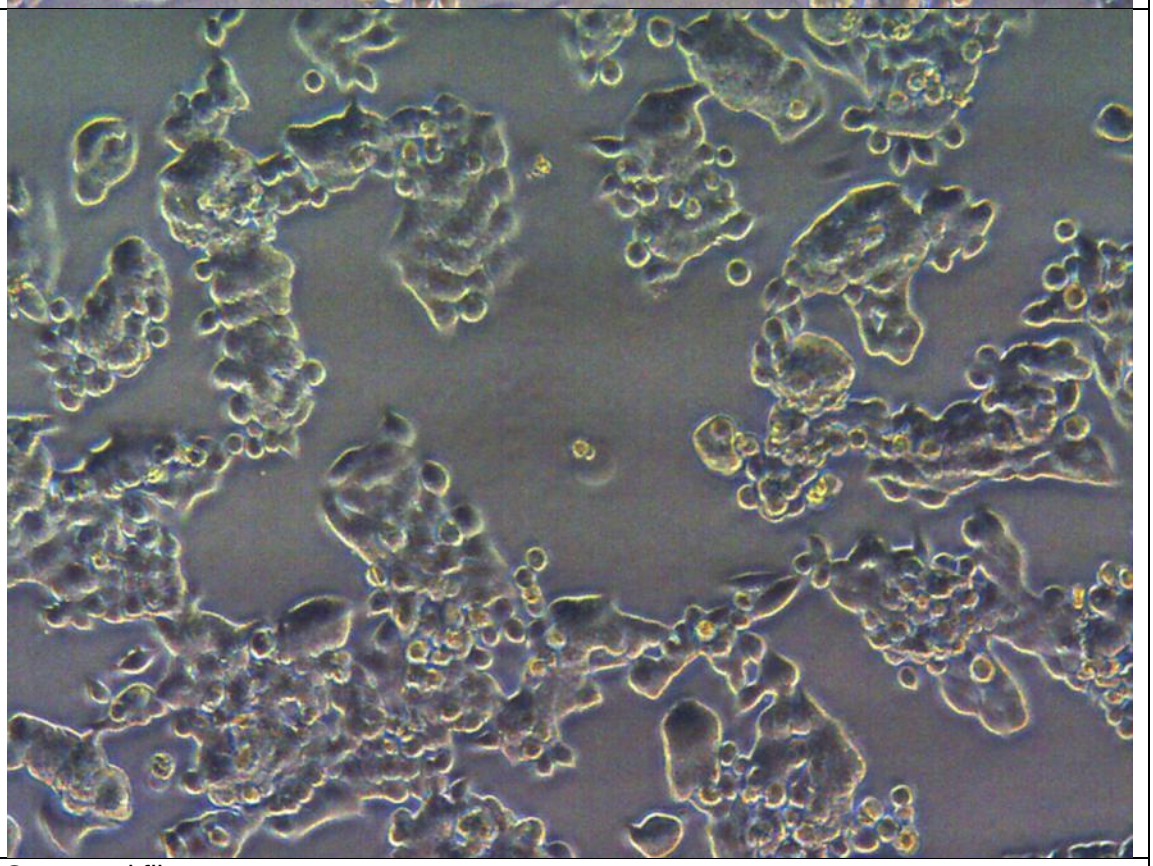
620 0h 1	

620 24h Cntlr 1	
620 24h Cntlr 2	

620 24h Cntlr 3	
620 24h Bhb 1	

620 24h Bhb 2	
620 24h Bhb 3	

620 48h Cntlr 1	
620 48h Cntlr 2	

620 48h Cntlr 3	
620 48h Bhb 1	
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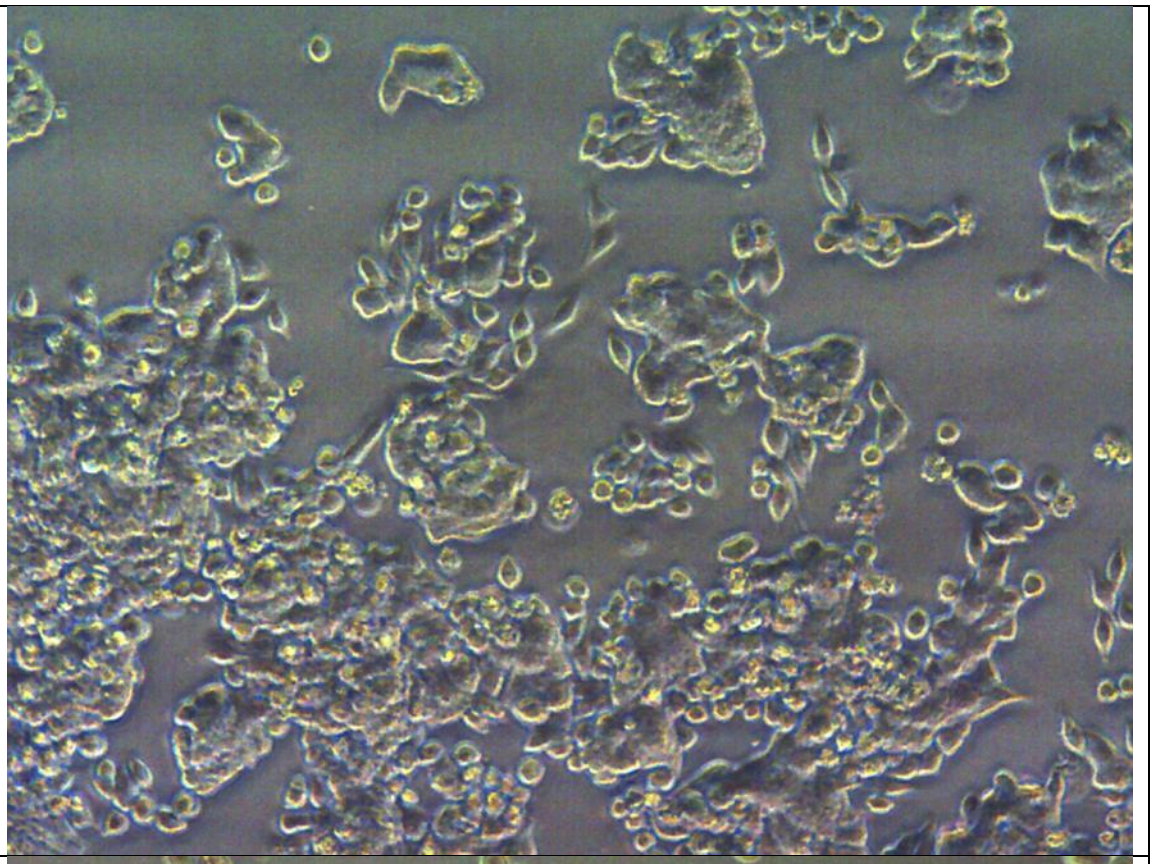
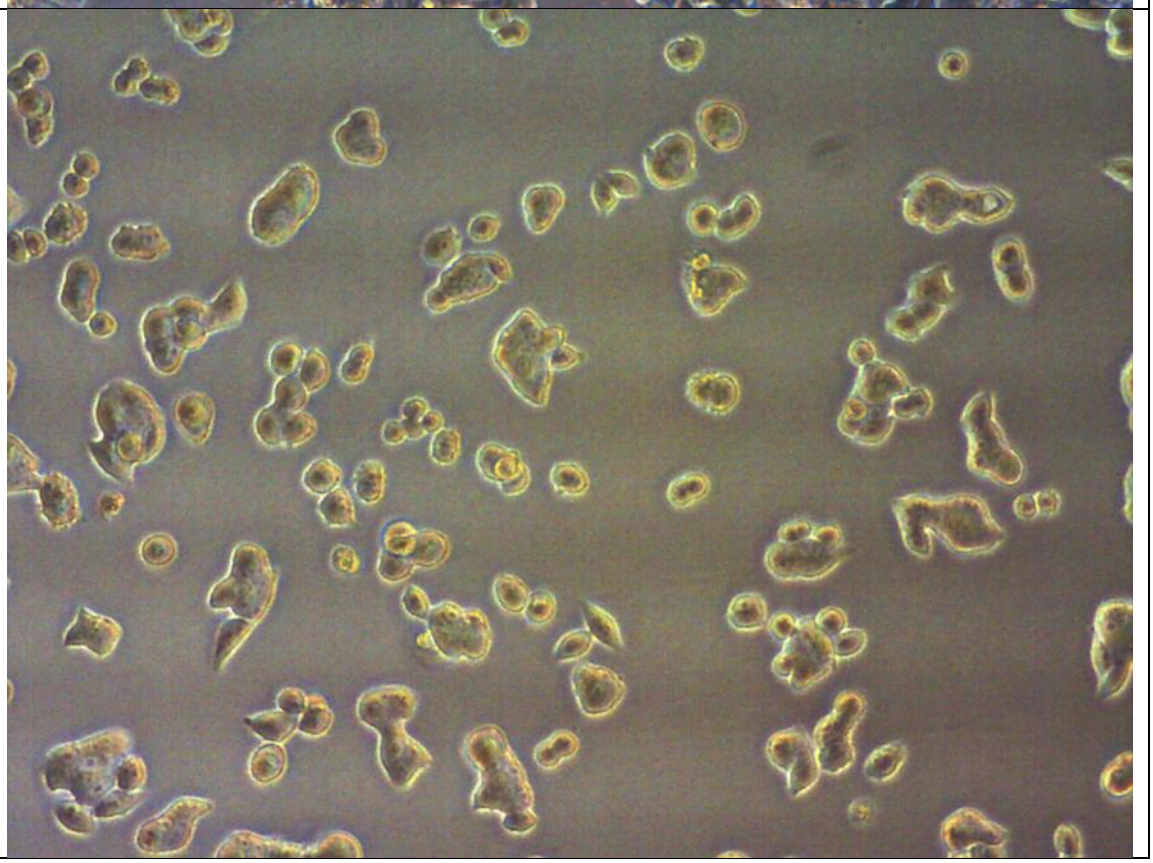
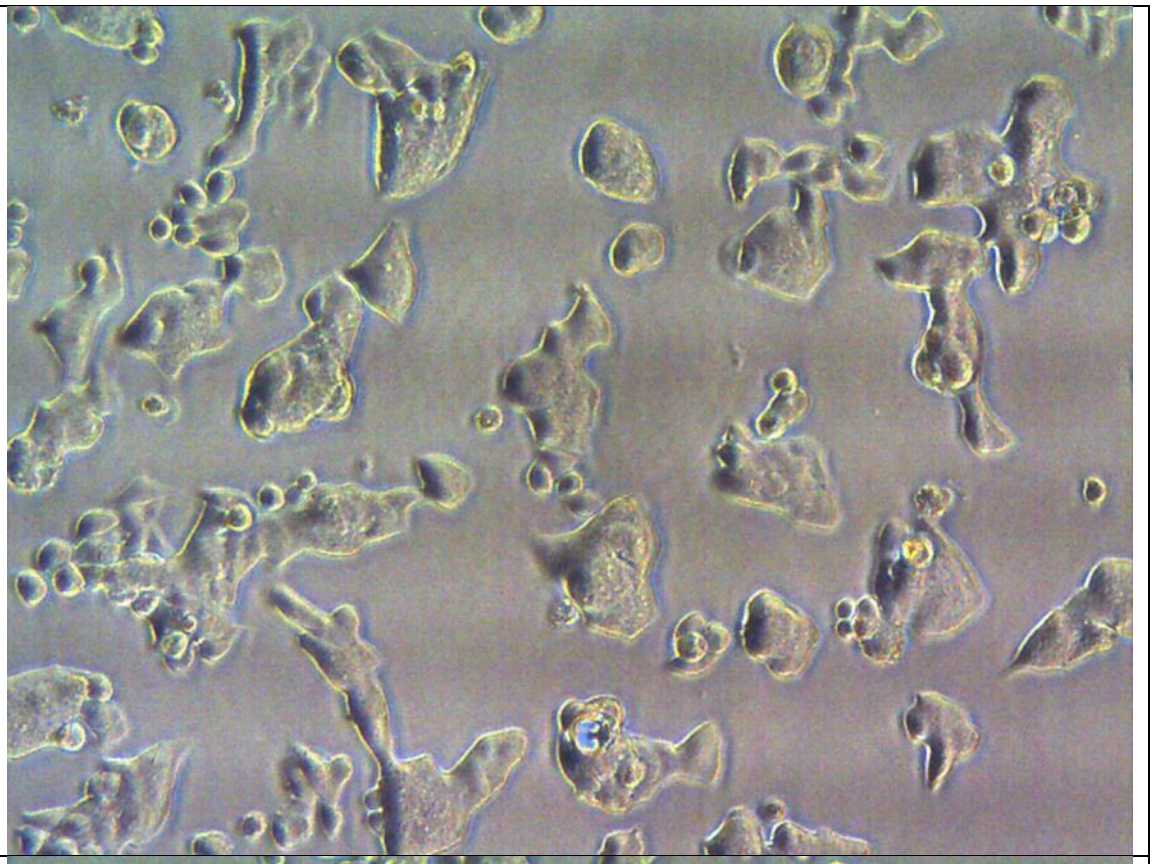
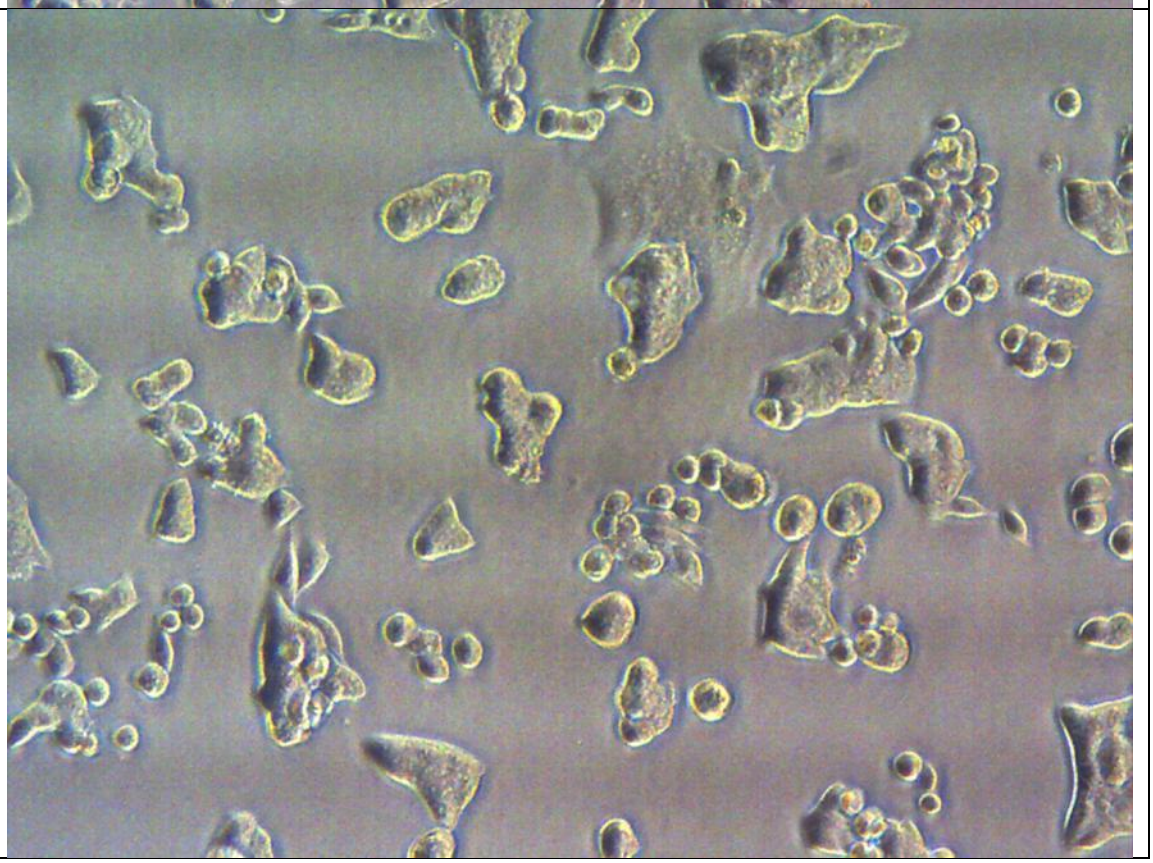
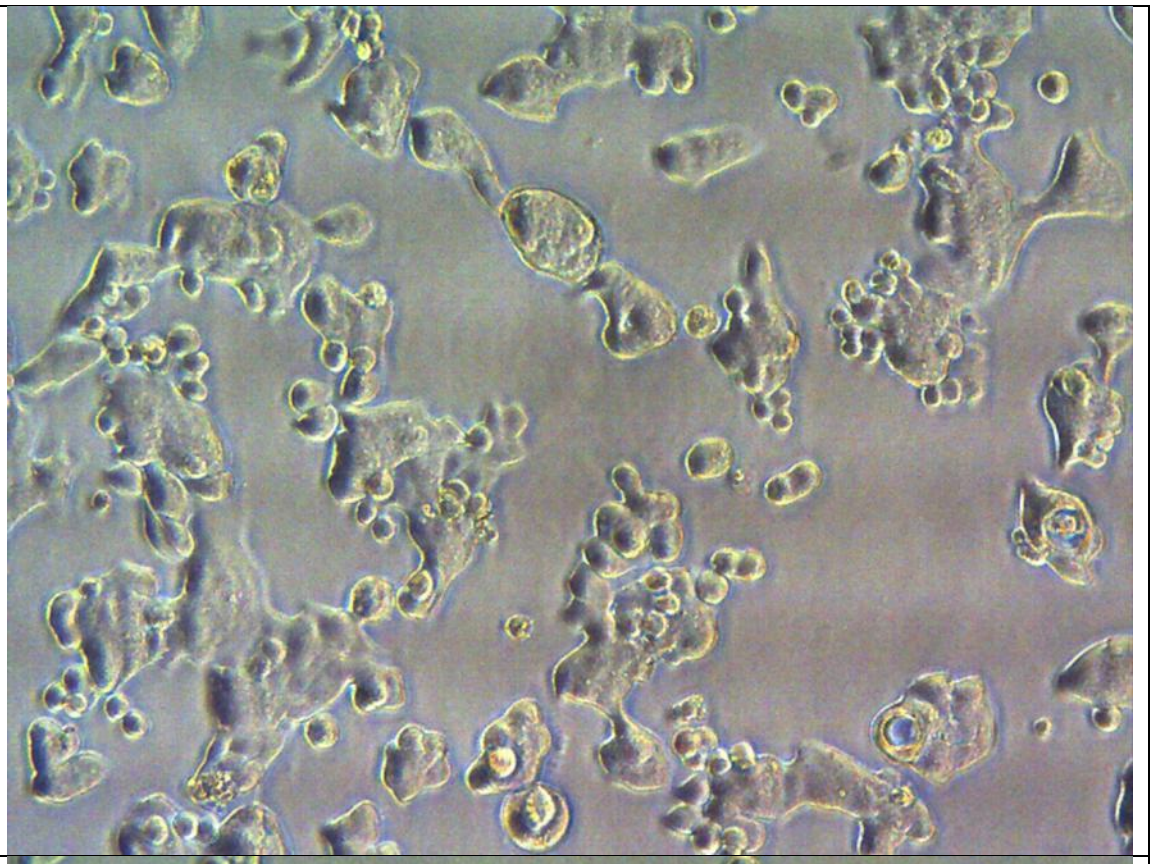
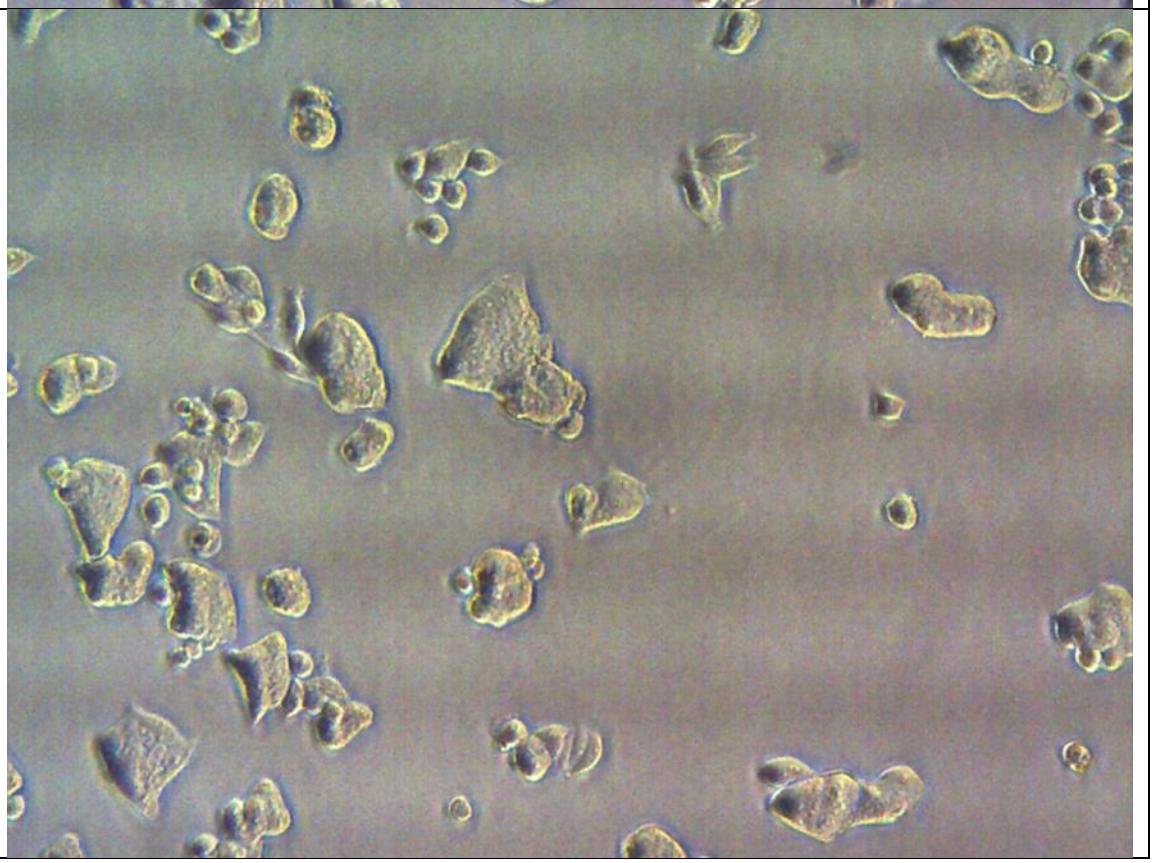
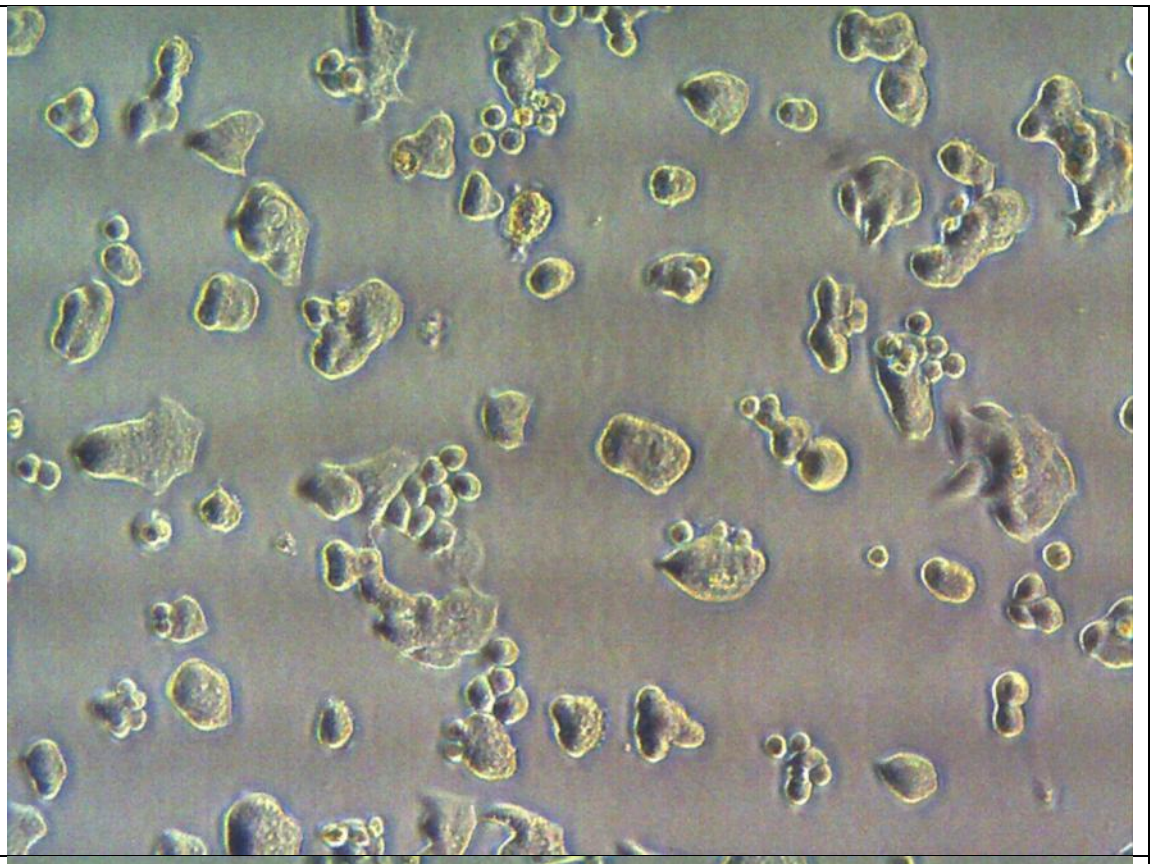
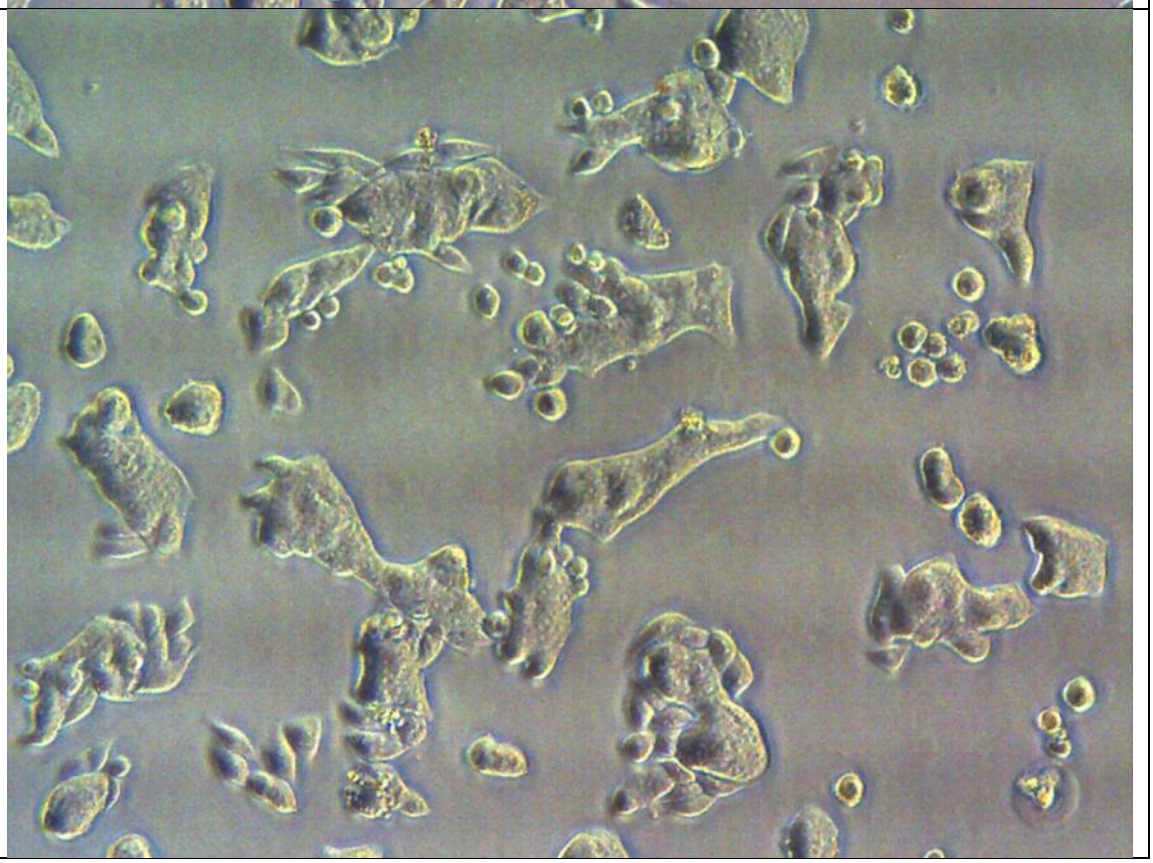
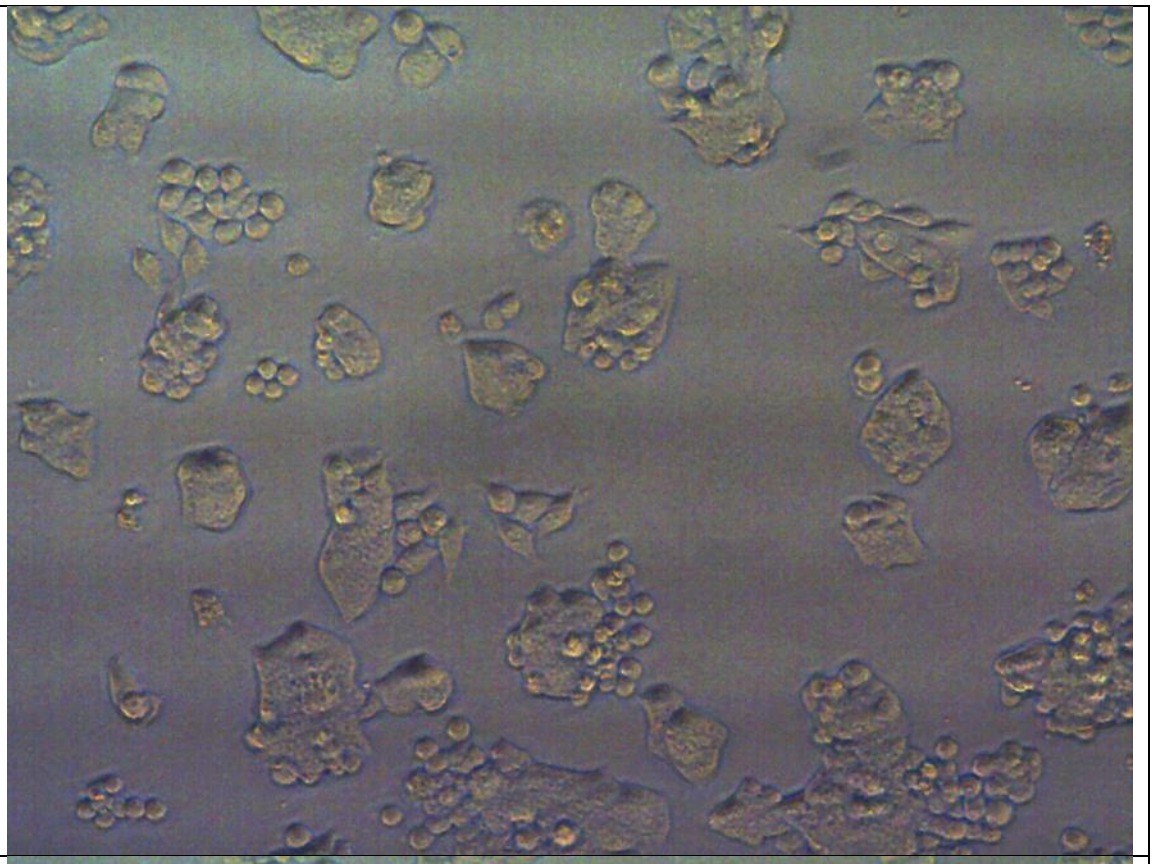
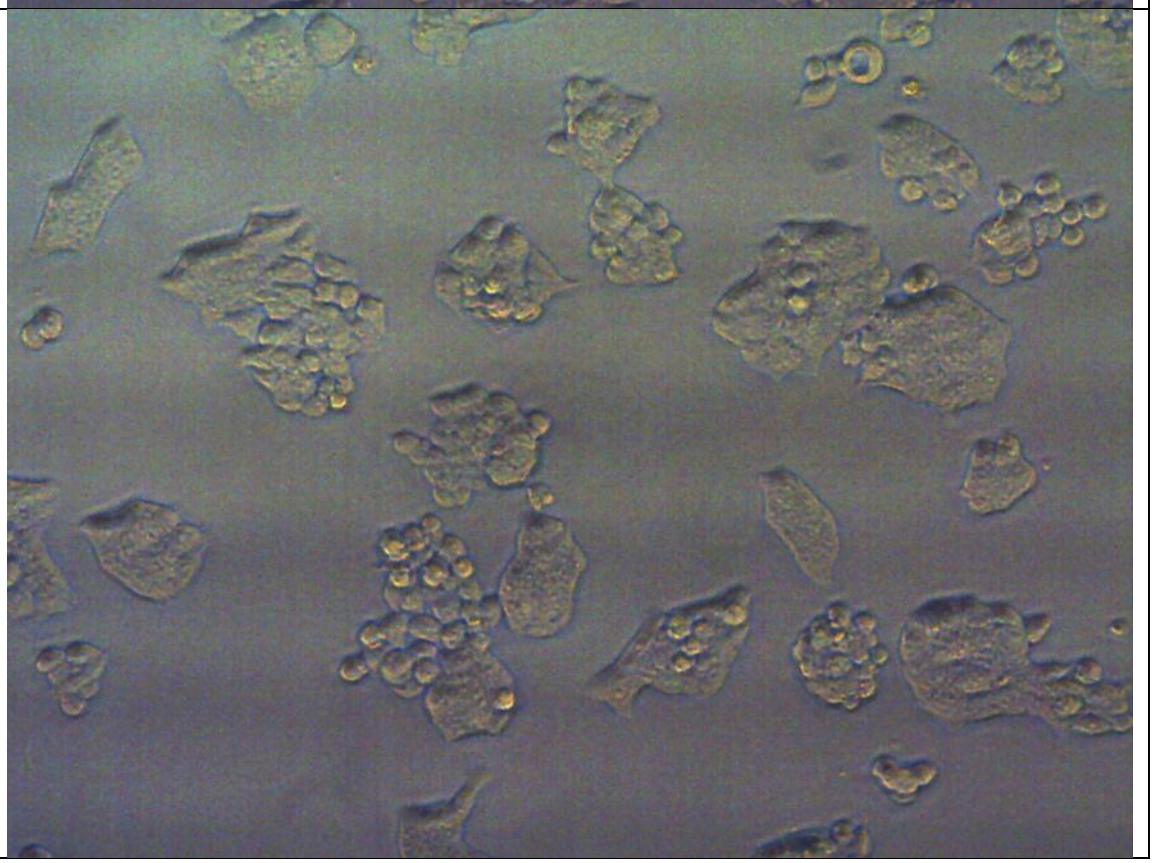
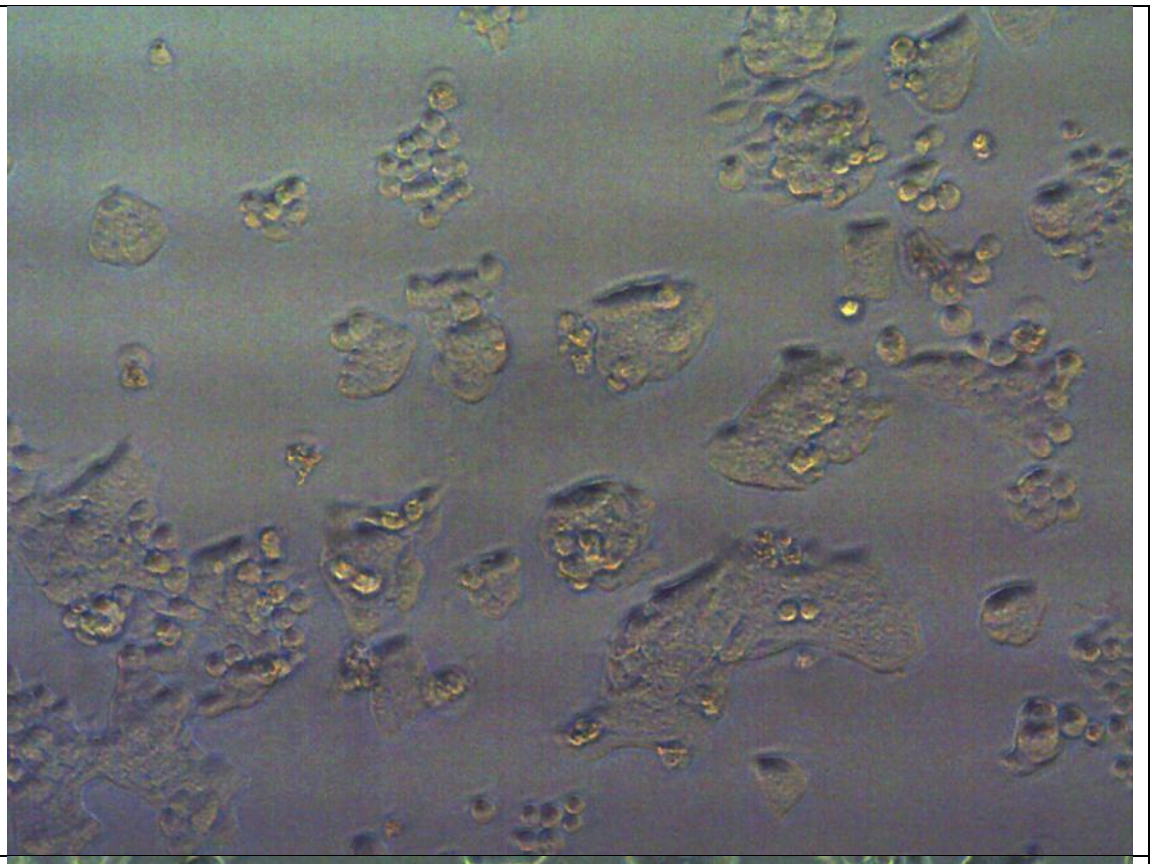
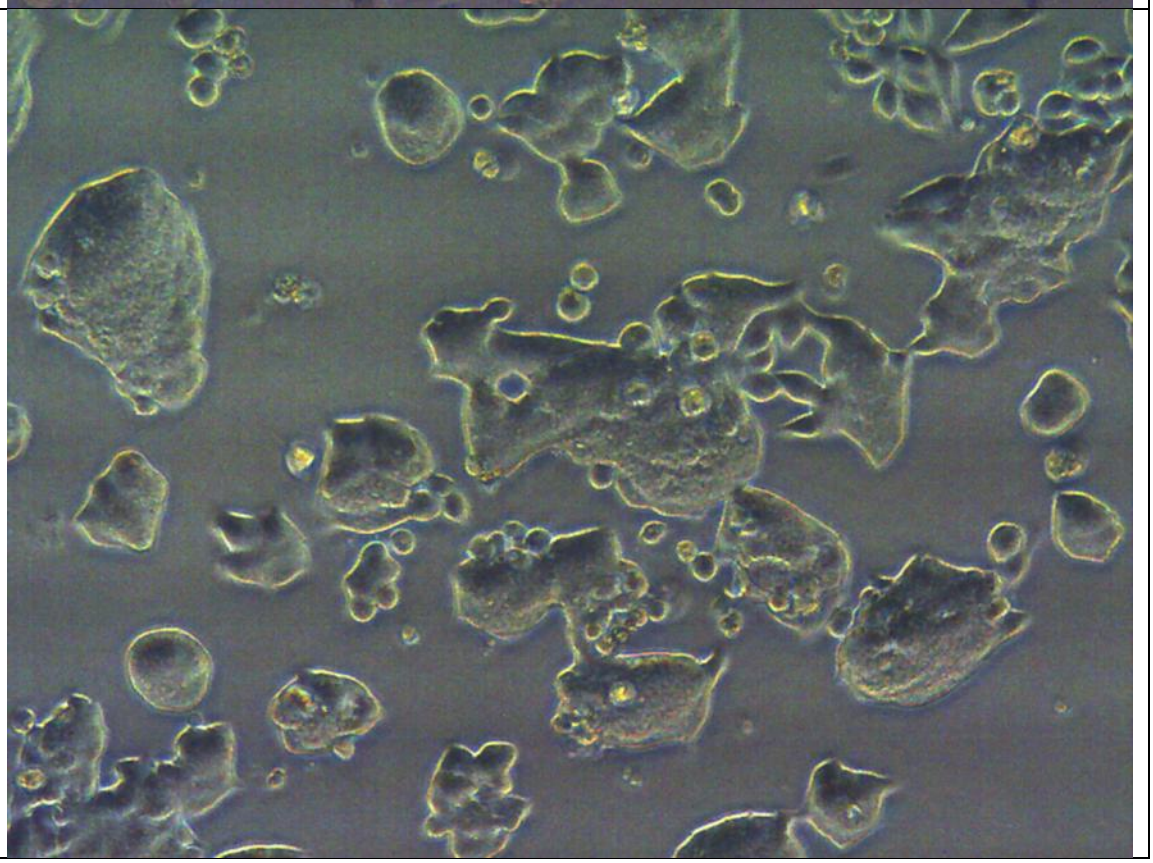
620 48h Bhb 3	
LiM2 0h	

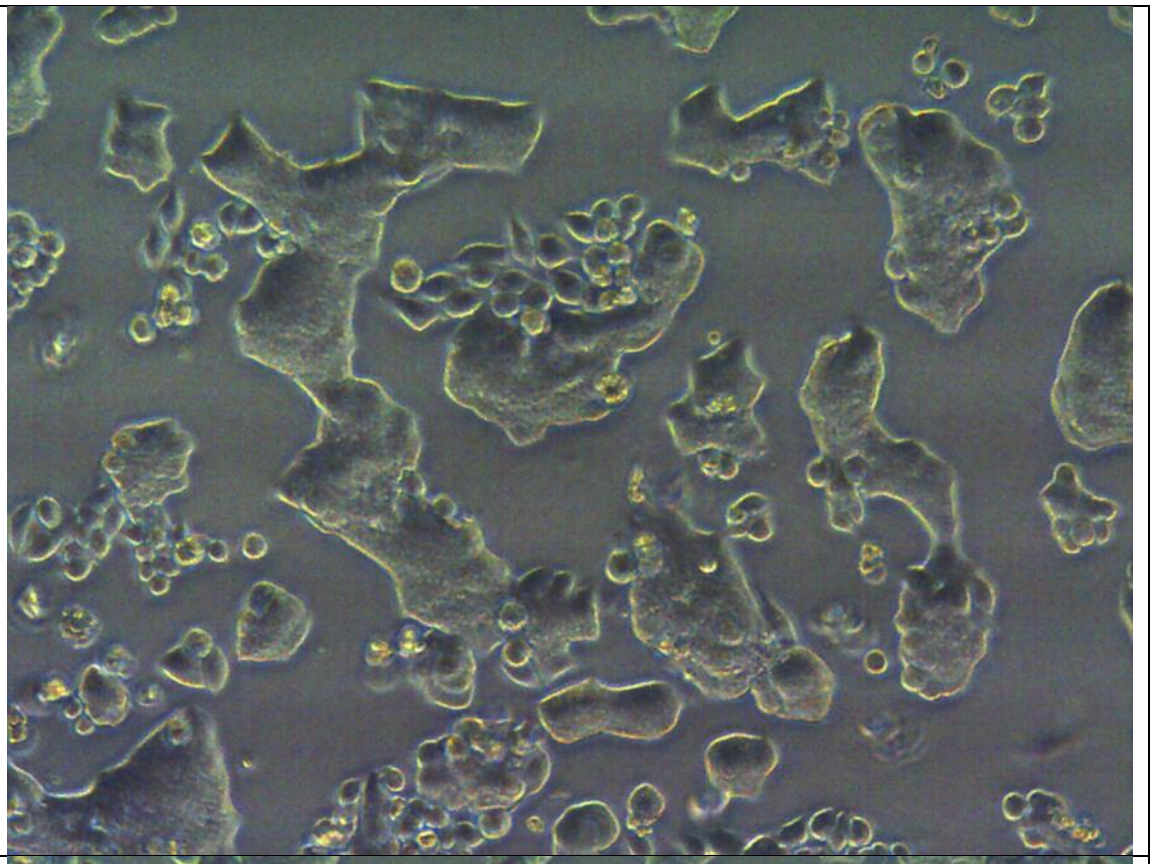
LiM2 24h Cntlr 1	
LiM2 24h Cntlr 2	

LiM2 24h Cntlr 3	
LiM2 24h Bhb 1	

LiM2 24h Bhb 2	
LiM2 24h Bhb 3	

LiM2 48h Cntlr 1	
LiM2 48h Cntlr 2	

LiM2 48h Cntlr 3	
LiM2 48h Bhb 1	

LiM2 48h Bhb 2	
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