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TRANSCRIPTIONAL CHANGES OF MYCOPLASMA CONTAMINATION IN GENE EXPRESSION STUDIES

BY

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ABSTRACT

Mycoplasma, the smallest self-replicating microorganism, is one of the most significant problems in the cell-culture field. It characterized by the absence of the cell wall, causing resistance to commonly used antibiotics in cell culture. The frequency of mycoplasma contamination has reached up to 35% worldwide, while the incidence found to be higher (42%) in Qatar. Numerous gene-expression studies have shown that mycoplasma dis-regulates thousands of genes on cultured cells causing unsafe biological products and erroneous experimental results. Therefore, it is critical to ensure that transcriptional changes due to mycoplasma are eliminated and gene-expression profile is restored to normal level after anti-mycoplasma treatment, to avoid biased results.

For this purpose, first, we developed a survey to investigate the incidence of mycoplasma contamination in cell-cultures and most common methods used for mycoplasma detection and elimination in research laboratories in Qatar. Second, we used next-generation sequencing technology (illumina 4000 Hi-seq) to investigate the effect of mycoplasma on gene-expression profile of cultured cells and the ability of antibiotic treatment in reversing these changes.

Results showed that number of dis-regulated genes in contaminated cells is increasing with the increase of sub-culturing. Affected genes were mainly related to cell death and survival and cell-to-cell signaling and interaction. Interestingly, genes dis-regulation was not only due to mycoplasma contamination, antibiotic treatment also

contributed to the effect. Unlike the effect of mycoplasma, antibiotic treatment showed a shrinking pattern on the number of dis-regulated genes.

Findings of this study concludes that mycoplasma contaminated-cells should not be further used or processed, specially for gene-expressions studies, due to the significantly dis-regulated genes as consequence of either mycoplasma or antibiotics effects.

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LIST OF ABBREVIATIONS

16S mRNA	16S Messenger Ribonucleic Acid
16S rRNA	16S ribosomal Ribonucleic Acid
6-MPDR	6-methylpurine deoxyriboside
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
bp	Base pair
CFU/ml	Colony-Forming Unit per Milliliter
CHO	Chinese hamster ovary cells
CML	Chronic Myelogenous Leukemia
CO2	Carbon Dioxide.
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl Slfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay.
ESC	Embryonic Stem Cell
FAD	Flavin Adenine Dinucleotide
FDR	Fouls Discovery Rate
GSA	Gene Specific Analysis

HEK 293	Human Embryonic Kidney
Hela	Cell line was derived from cervical cancer of Henrietta Lacks.
hg19	Human Genome version 19
HGE-3 cells	Human granulocytic ehrlichiosis
HiSeq	High-Throughput Sequencing
HMC	Hamad Medical Corporation
HUVECs	Human Umbilical Vein Endothelial Cells
IPA	Ingenuity Pathway Analysis
IOM	International Organization for Mycoplasmology
ISO	International Organization for Standardization
MRA	Mycoplasma Removal Agent
NCBI	National Center for Biotechnology Information
NGS	Next-Generation Sequencing
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PNAS	Proceedings of the National Academy of Sciences of the United States of America
PPLO	Pleuropneumonia-like organisms
QBRI	Qatar Biomedical Research Institute
QCRC	Qatar Cardiovascular Research Center
QSTP	Qatar Science and Technology Park
QU	Qatar University

RNA-seq	RNA Sequencing.
RNA	Ribonucleic Acid
RNase	Ribonuclease
RT-PCR	Real Time - Polymerase Chain Reaction
S2B2	Drosophila S2 cells.
SKOV3	Human Ovarian Adenocarcinoma
UKCCCR	United Kingdom Coordinating Committee on Cancer Research
WCM-Q	Weill Cornell Medicine- Qatar

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CHAPTER 1: INTRODUCTION

Cell-culture has become the most common biomedical model in biology and medical research (Zalai et al., 2015). Cell culture contamination with mycoplasma, the smallest self-replicating microorganism, is one of the most serious and significant problems in the cell-culture field (Laleh et al., 2011).

Mycoplasma belongs to the *Mollicutes* class, with a unique feature of cell wall absence (Uphoff et al., 2014), making them resistant to most common antibiotics (streptomycin and penicillin) used in cell-culture (Miller et al., 2003; Uphoff et al., 2011; Rottem et al., 2012). Because of their small size, mycoplasma are able to pass through the small pores (0.2 μm) of media sterilizing filters (Young et al., 2010). Cell contamination with mycoplasma cannot be easily detected under light microscopy and also they do not yield turbid growth (Nikfarjam et al., 2012). The three major sources of mycoplasma contamination in cell-culture are: 1) cross-contamination from infected cultures, 2) contaminated culture reagents (e.g. serum and trypsin), and 3) laboratory personnel carrying certain type of mycoplasma, e.g. *M. orale* or *M. fermentans* (Hay et al., 1989). Several effective kits and methods have been established to detect and eliminate mycoplasma contamination like polymerase chain reaction (PCR) assay and antibiotics, respectively.

The high incidence of mycoplasma contamination among primary and secondary eukaryotic cell lines (up to 87% of cell lines) represents a significant problem in research, diagnosis, therapy, and industrial production (Rawadi et al., 1995). The first mycoplasma contamination incidence was reported in the 19th

century (Robinson et al., 1956), since then, the frequency of mycoplasma contamination in cell-culture has been extremely discussed in the literature (McGarrity et al., 1984; Hay et al., 1989; Nikfarjam et al., 2012. Previous reports showed high incidences of mycoplasma contamination reaching up to 35% globally, while in some countries like Japan and Argentina reported higher (65% and 80%) (Uphoff et al., 2012; Uphoff et al., 2014).

The contamination of cell-culture by mycoplasma has diverse and comprehensive consequences, ranging from unsafe biological products, to erroneous experimental results (Miller et al., 2003). Due to the lack of amino acid biosynthesis genes, mycoplasma competes with its' host cell nutrients and biosynthetic precursors (Razin et al., 2002), leading to DNA, RNA and protein synthesis alterations (McCormack et al., 1993). Using conventional gene qualification methods and microarray platforms, several gene-expression studies have shown that mycoplasma contamination significantly up regulates and down regulates thousands of genes like oncogenes, tumor suppressors, cytokines and growth factors (Miller et al., 2003; Zhang et al., 2006). However, none of these studies investigated the reversibility of these transcriptional changes after antibiotic treatment of contaminated cells.

1.1 HYPOTHESIS

Antibiotic treatment of mycoplasma contamination in cell-culture will not eliminate transcriptional changes caused by the mycoplasma, neither restore cell's gene-expression profile to normal expression.

1.2 Significance Of The Problem

In gene-expression studies, it is critical to ensure that transcriptional changes are originated from experimental condition, but not due to other artifacts or contaminants. Therefor, it is important to eliminate mycoplasma contamination in cell-culture and to ensure that gene-expression profile is restored to normal level after antibiotic treatment, to avoid biased results. High throughput RNA-sequencing platform will be used, to investigate the transcriptional changes caused by mycoplasma contamination in cell-culture and assess the reversibility of these transcriptional changes after antibiotic treatment of contaminated cells. All the work will be guided by a survey developed to investigate the incidence of mycoplasma contamination in cell-cultures and the techniques frequently used for the detection and elimination of mycoplasma in research laboratories in Qatar.

1.3 Research aims and objectives

The aim of this study is to investigate the validity of using mycoplasma-contaminated cell-culture post antibiotic treatment in gene-expression studies.

Objectives:

1- Develop a survey to investigate the incidence of mycoplasma contamination in cell-cultures and the techniques frequently used for the detection and elimination of mycoplasma in research laboratories in Qatar.

2- Investigate the effect of mycoplasma in cell morphology and gene-expression profile.

3- Assess the ability of anti-mycoplasma treatment (antibiotics) in reversing gene-expression and morphological changes caused by mycoplasma.

CHAPTER 2: REVIEW OF THE LITERATURE

2.1 MYCOPLASMA

To explore the altered appearance of plant cell cytoplasm, resulting from fungus like microorganisms infiltration, Albert Bernhard Frank was the first to use the term mycoplasma in 1889 (Mehrotra et al., 2015). Mycoplasma is the derivative of Greek μυκής, mykes (fungus) and πλάσμα, plasma (formed). Since the work of Edward and Freundt in 1950s the original term has been extensively used although it was substituted by *Pleuropneumonia-like organisms* (PPLO) (Edward et al., 1936).

Due to the absence of cell wall around the plasma membrane, mycoplasma is grouped to the genus of bacteria on which antibiotics do not exert prominent effect (such as penicillin). The inactivity of antibiotics is attributed to the lack of cell wall, which is the target of these antibiotics (such as beta-lactam antibiotics). Majority of mycoplasma including *M. pneumonia* have been found to be human pathogens, although, these can be parasitic in nature as well (Nocard et al., 1990).

The first mycoplasma species found in human, now is called *M. hominis*, was isolated by Edsall and Dienes from a gland abscess (Tully et al., 1996; Waites et al., 2006). This discovery was in 1956 in cell cultures, after the finding of mycoplasma growth in negative control in cell cultures, (Robinson et al., 1956).

Cell cultures, animals and human have been found to be the host of mycoplasma, where it can live as pathogens or commensally (the human is known to be used as primary host by approximately 16 - 20 species). Mycoplasma exhibit tissue host specificities and it has the ability to survive for prolonged period of time, and

increase in number after entry into cell. Their ability to avoid the detection by host immune system is attributed to the development of mimicry against host antigens as observed by mycoplasma over time (Razin et al., 1998, Rottem et al., 2003).

2.2 TAXONOMY OF MYCOPLASMA

Humans are known to be use as primary host by at least 16 out of more than 180 species of mycoplasma discovered to date. They form one of the eight sub-divisions (or genera) that constitute the *Mollicutes* class.

The close relationship of mycoplasma to *Lactobacillus*, *Bacillus* and Gram-positive *Clostridia* has been clearly demonstrated by phylogenetic studies involving the sequence analysis of 16S mRNA. Because of phenotypic properties and sequence similarities, mycoplasma, however, have been reclassified into three groups *spiroplasma*, *pneumonia* and *homini* (Figure 2.1) (Weisburg et al., 1989; Drexler et al., 2000).

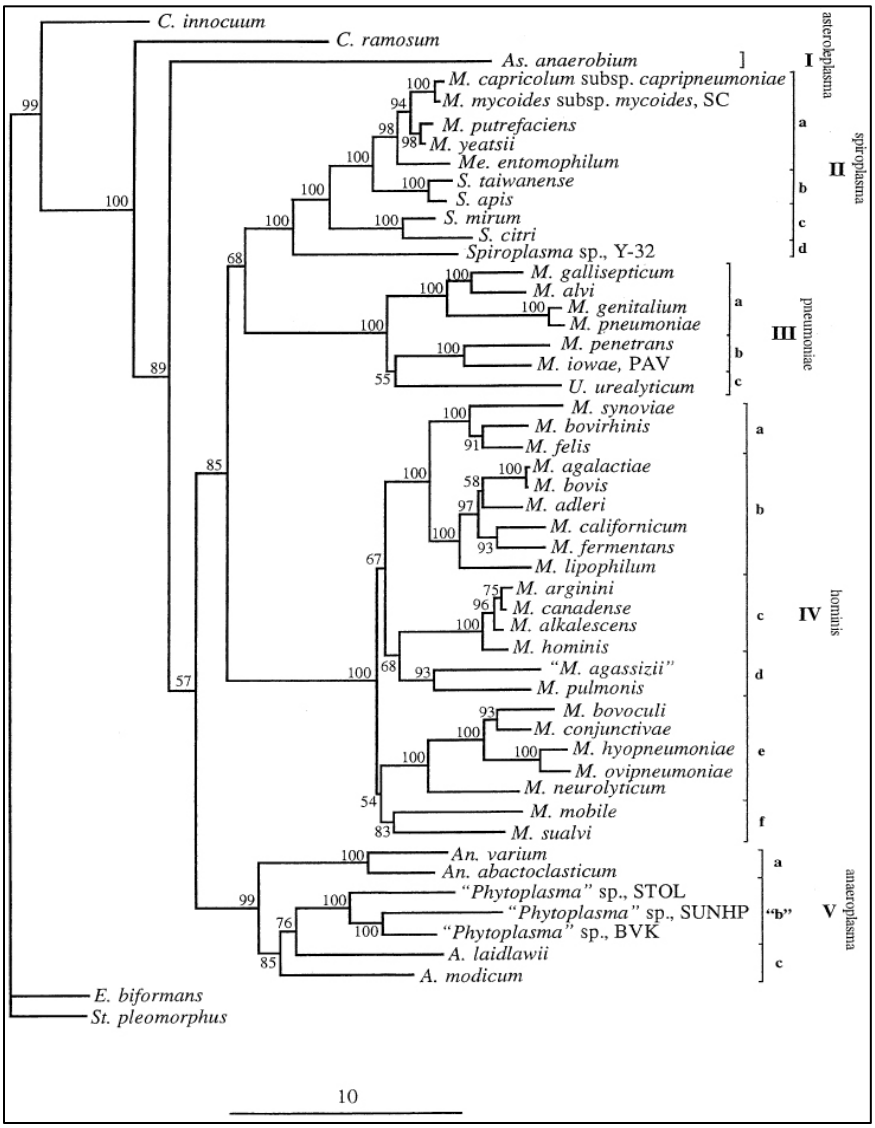

Figure 2.1. Phylogenetic tree of mycoplasmas based on 16S rRNA sequences. Showing more than 180 species of mycoplasma discovered to date, which are belong to *Mollicutes* class, classified into three main groups: *spiroplasma* (II), *pneumonia* (III) and *homini* (IV) (Razin et al., 2006)

2.3 BIOLOGY OF MYCOPLASMA

Generally, to date, mycoplasmas are the smallest self-replicating organisms and found to be between 0.1 to 0.8 µm (Razin *et al.,* 1978; Drexler *et al.,* 2000). They have been discovered to be pleomorphic and are able to demonstrate a variety of shapes like pear, flask, pill and filamentous amongst others (Kim *et al.,* 1966*;* Razin *et al.,* 1998). Additionally, they are characterized by absence of the cell wall, instead they are surrounded by 3 layers of lipoprotein membrane (Figure 2.2), which make them immune against the effect of antibiotics that target cell wall synthesis in order to kill the cell (Razin *et al.,* 1998; Rottem *et al.,* 2003). This may be because they lack many of the enzymatic pathways such as wall production pathway, a functional tricarboxylic acid cycle as well as a cytochrome mediated electron transport system (Dybvig *et al.,* 1996; Drexler *et al.,* 2000).

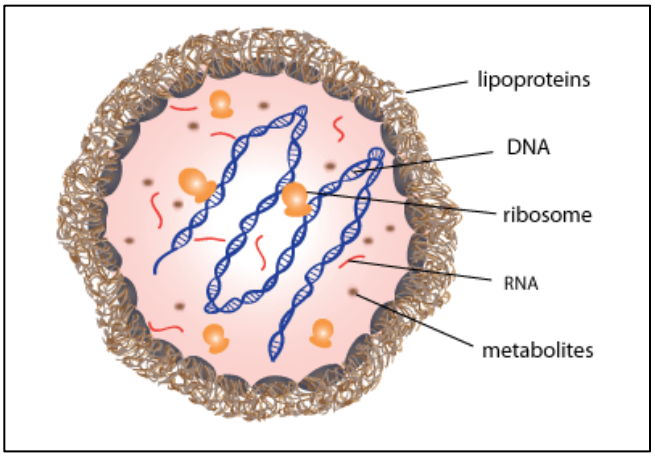

Figure 2.2. **Cell structure of mycoplasma**. Showing the main components of mycoplasma and the three layers of lipoprotein membranes that replacing the cell wall (www.invivogen.com)

Mycoplasma have been known to form colonies after 3 to 5 days on a solid agar with a characteristic 'fried egg' appearance (Figure 2.3) with a diameter of up to 400 μm, while in other cases they have been observed to grow slowly and not be recognizable for up to 3 weeks or more (Drexler *et al.,* 2002).

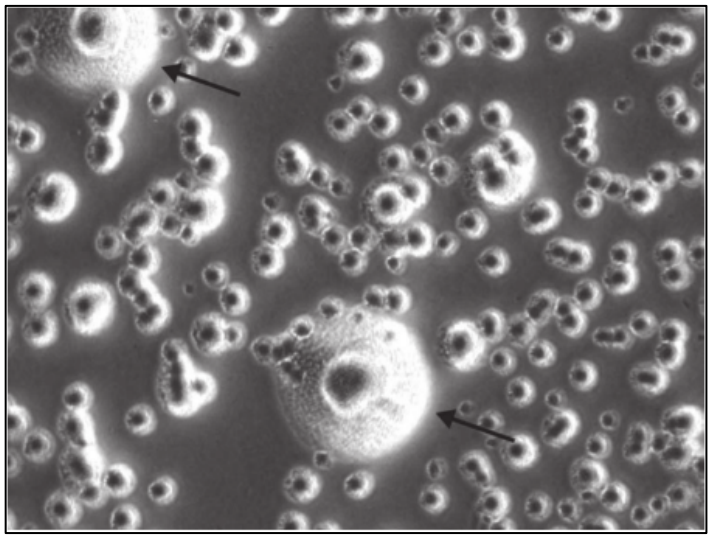

Figure 2.3. Typical mycoplasma colonies on agar. Showing the classic 'fried egg' appearance of most of mycoplasma growth under the microscope, 3 to 5 days (Young *et al.,* 2010)

Owning a double strand DNA, investigations showed that mycoplasma uses a binary fusion method to divide. It is also noted that the division of cytoplasm and the replication of mycoplasma genome are following an asynchronous rather than synchronous replication. Which explain the phenomena of filament formation and

cocci chains transformation of mycoplasma during division process (until division is complete) (Razin *et al.,* 1998; Drexler *et al.,* 2000). However, they have a relatively long lag phase with a generation time of between 1 to 9 hours (Drexler *et al.,* 2000).

The genome of mycoplasma has been found to range from 580 kb to 2.22 kb (*M. genitalium* and *Spiroplasma* respectively), with GC content up to 33 mol%. Unlike other bacteria, the genome size of mycoplasma considered being very small, it consists of around 500 genes (one-fifth size of bacterial genome) (Dybvig *et al.,* 1996; Razin *et al.,* 1998; Drexler *et al.,* 2000; Rottem *et al.,* 2003). Is has also been found to possess inefficient energy yielding pathways (Dybvig *et al.,* 1996; Drexler *et al.,* 2000).

Mycoplasma is characterized as either non-fermentative or fermentative on the basis of their potential to produce ATP. In non-fermentative mycoplasma, ATP is produced by arginine hydrolysis using arginine dihydrolase pathway, whereas the carbohydrate fermentation by using glycolytic pathway is responsible for ATP production in fermentative Mycoplasma. However, both metabolic pathways have been observed to be involved in ATP production in some mycoplasma such as *M. fermentans*. On the basis of enzymatic ATP production found in both groups of mycoplasma (non-fermentative and fermentative), an alternative pathway for ATP production was suggested as a consequence of above discoveries and observations (Razin et al., 1978 & 1998; Drexler et al., 2000).

2.4 MYCOPLASMA IN CELL CULTURES

Remaining virtually undetectable under a conventional microscopy, mycoplasma has been observed to be very easily contaminating cell-cultures under regular laboratory conditions. It is able to present itself without causing any visible effects and can therefore remain relatively undetected. Due to its plasticity it is able to pass through some microbiological filters that measure 0.22 μm or less (Drexler *et al.,* 2000). In addition, it is able to show resistance to most common cell-culture antibiotics due to the fact that it is Gram-negative and lack of the cell wall, as mentioned above, antibiotics typically tend to target for cell wall destruction (Drexler *et al.,* 2000; Uphoff *et al.,* 2005).

Due to mycoplasma persistent nature and resistance to dehydration, contaminated cell lines as well as reagents, laboratory equipment and individuals can very easily provide a source of mycoplasma contamination (McGarrity *et al.,* 1985; Tully *et al.,* 1996; Drexler *et al.,* 2000 & 2002).

Typically, mycoplasma can grow very well in cell cultures, but mycoplasma infection is very much dependent on the dosage and concentration and type of cells involved. Given the right conditions, within three to five days, individual mycoplasma cell has been observed to grow to million CFU/ml (Colony Forming Unit per milliliter) in contaminated cell-culture with a titers of millions to hundred millions CFU/ml. Under the microscope, it has been observed that around 100 to 1000 mycoplasmas will attach themselves to each host cell whenever there is a heavy or concentrated infections (Drexler *et al.,* 2000).

2.5 INCIDENCE OF MYCOPLASMA CONTAMINATION IN CELL CULTURES

The frequency of in vitro mycoplasma contaminations can vary worldwide depending on the detection methods used, geography, number and concentration of cell cultures examined (McGarrity *et al.,* 1985; Tully *et al.,* 1996). In comparison to the contamination rates of 35% and 15% in animal and human cell lines, primary cell cultures and their sub-cultivated passages are less frequently contaminated with reported evidences of only 1% and 5%, respectively (Table 2.1) (Drexler et al., 2002). In the beginning of 1990s, over twenty thousands cell cultures tested by United States Food and Drug Administration (USFDA), 15% of cells were found to be mycoplasma contaminated (Rottem *et al.,* 1993). In addition, 70% and 28% cell lines were mycoplasma positive in Argentina and Germany, respectively (Coronato *et al.,* 1990; Drexler *et al.,* 2002). Recently, RNA-sequencing data showed contamination rate of 11% and 7% (Olarerin-George and Hogenesch, 2014; Langdon, 2014).

Table 2.1. Incidence of mycoplasma contamination (Drexler and Uphoff, 2002)

Cell type	**Incidence**
Continuous human or animal cell lines	15–35%
Early passage cell cultures	5%
Primary cell cultures	1%

Due to the use of contaminated serum, animal mycoplasma species such as *M. arginine* and *M. hyorhinis* were thought to be the most commonly found species of mycoplasma in cell cultures (McGarrity *et al.,* 1985). However, current studies indicates that *M. orale* (a species that prefers humans as host) is the most commonly found species with a frequency rate of 20% to 40% (possibly as a result of contamination from individuals), followed by *M. hyorhinis* (10% to 40%), *M. arginine* (30%) and *M. fermentans* / *M. hominis* (10% to 20%) (Table 2.2) (Drexler *et al.,* 2002). However, the incidence of multiple infections, that involve two mycoplasmas species or more, can be as low as 7% or higher up to 60% (Uphoff *et al.,* 2002; Timenestsky *et al.,* 2006).

Table 2.2. Most common Mycoplasma species (Drexler and Uphoff, 2002)

Mycoplasma species	**Source**	**Incidence**
M. *orale*	Human	20-40%
M. *hyorhinis*	Swine	10-40%
M. *arginini*	Bovine	20-30%
M. *fermentans*	Human	10-20%
M. *hominis*	Human	10-20%
A. *laidlawii*	Bovine	5-20%

2.6 EFFECTS OF MYCOPLASMA CONTAMINATION

It has been observed that under laboratory conditions, mycoplasma can affect various cell parameters such as growth and proliferation, morphology, metabolism, genome and antigenicity of contaminated cells (McGarity *et al.,* 1984 & 1985; Drexler *et al.,* 2000 & 2002).

Effect of mycoplasma is very much dependent on the particular species of mycoplasma, cell type to be infected, conditions of culture, intensity and duration of infection (Drexler *et al.,* 2002).

With regard to growth, morphology and metabolism cytopathic effects of mycoplasma on cells have been observed as a result of cell component consumption by mycoplasma. Mycoplasma consumes cell supplements, nutrients and cell metabolism components e.g. nucleic acid precursors, amino acids, vitamins, cholesterol and lipids. These compounds are normally used up very rapidly due to the fact that they have a low metabolic rate, an inefficient energy gain and because of a possible high number of mycoplasma present in the cell culture. Furthermore, the production of hydrogen peroxide (H_2O_2), low pH environment and the exhaustion of arginine plus other nutrients have also been noted and these have been found to induce increased granularity, cytotoxicity and lysis resulting in cell death (McGarity *et al.,* 1985; Drexler *et al.,* 2000). Mycoplasma also affect RNA / DNA synthesis, change the proteins level and directly affect cell metabolism resulting in cell degeneration that shows pyknotic nuclei, marginated chromatin and segregated (Stanbridge *et al.,* 1971; Drexler *et al.,* 2000 & 2002).

In addition, mycoplasma has been observed to affect the proliferation and growth rate of contaminated cells, causing irregular and arrested growth patterns with other abnormalities such as rounded degenerated cells and detachment of monolayers. However, the introduction of certain nutrients to culture could reverse some effects while others remain irreversible (Drexler *et al.,* 2000).

With regard to effects on genome, mycoplasma have the ability to degrade host cell DNA (Paddenberg *et al.,* 1998) and dis-regulate hundreds of host genes, mainly effecting genes associated with immune responses, cell-cycle regulation and signal transduction pathways (Hopfe *et al.,* 2013). Mycoplasma infections have been observed to cause a reduction of the number of chromosomes present, and cause variations in the appearance of new chromosomes. But the main effects of mycoplasma on the genome are chromosomal aberrations such as chromatid breaks, achromatic gaps, rearrangements and translocations, dysenteric and pulverization (Barile *et al.,* 1979, McGarrity *et al.,* 1985, Drexler *et al.,* 2000).

Regarding the effects on antigenicity, in vitro, mycoplasma can affect hematopoietic cells by stimulating, or conversely, inhibiting their activation, propagation and differentiation by enhancing the immunoglobulin secretion of B-cells and the induction of cytokines and growth factors (McGarity *et al.,* 1985; Razin *et al.,* 1998; Drexler *et al.,* 2000). All these effects are because of the way that mycoplasma interacts with cells and cause changes in the composition of the cell membrane, which subsequently affects the expression of surface antigens and receptors (Drexler *et al.,* 2000). This can also directly influence viruses to propagate in the cultured cell. While

the non-fermentative mycoplasma, has the ability of arginine depletion, can negatively effect arginine dependent viruses propagation (such as Herpes simplex, adeno-viruses and vaccinia) (McGarrity *et al.,* 1985; Drexler *et al.,* 2000).

2.7 DETECTION OF MYCOPLASMA IN CELL CULTURES

The extensive use of cell cultures in medical research and in the manufacturing of biological products, all cells should be screened for the presence of mycoplasma before being used. This is due to the direct effects of mycoplasma on several biological cell parameters (as discussed above), which can ultimately cause misleading experimental results and contaminate intended biological products. So far, several techniques have been developed for the detection of mycoplasma in cell cultures (Table 2.3) (Drexler *et al.,* 2000).

Table 2.3. Selected Methods for Mycoplasma Detection (Drexler et al., 2000)

Microbiological culture	• Growth in liquid medium or Broth
	• Formation of typical small colonies on agar (Figure 2.3)
Electron microscopy	• Transmission/Scanning electron microscopy (Figure 2.4 and 2.5)
Biochemical assays	• Detection of adenosine phosphorylase activity (6-MPDR assay)
	• Enzymatic conversion of ADP to ATP detected by luciferase
	• Chromatographic detection of conversion of radioactively labeled uridine to uracil by Mycoplasmal uridine phosphorylase
Immunological assays	• Immunofluorescence
	• Enzyme linked immunosorbent assay (ELISA)
Molecular biological assays	• Liquid hybridization assay
	• Autoradiography (dot-blot) with Mycoplasma specific probes
	• Polymerase chain reaction (PCR) (Figure 2.6) and reverse transcription PCR
	• PCR-ELISA
	• Species-/genus–specific PCR primers or universal PCR primers
	• Gradient electrophoresis separation of labeled RNA
Microscopic detection assays	• Direct DNA fluorescent staining (DAPI, Hoechst 33258) (Figure 2.7)
	• Indirect DNA fluorescent staining with indicator cell line
	• Fluorescent in situ hybridization
Histological staining	• Histochemical stains and light microscopy
Protein analysis	

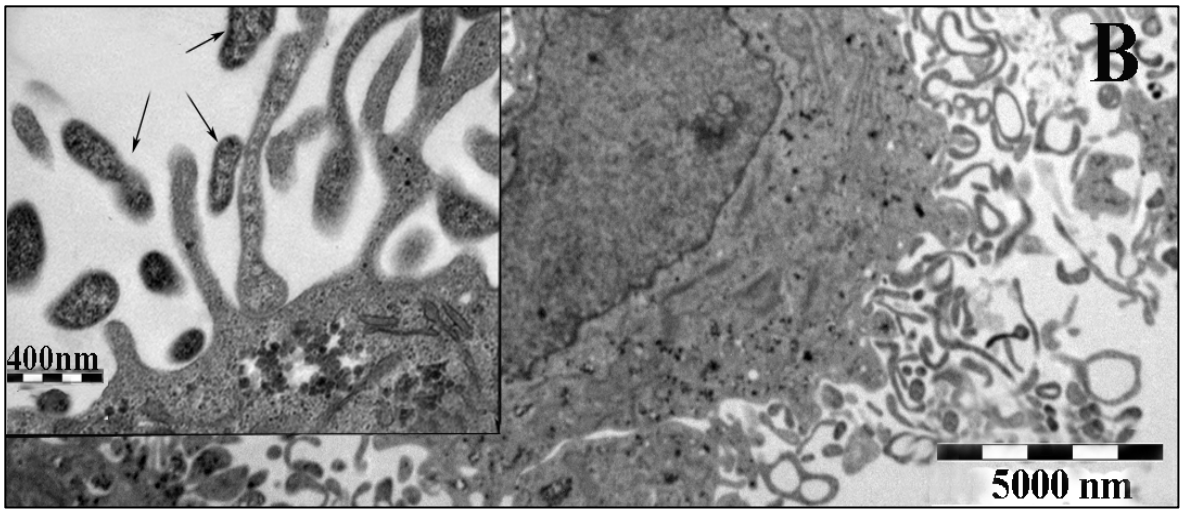

Figure 2.4. Transmission electron microscopy of melanoma cell culture infected by *M. hyorhinis*. Showing flask shaped mycoplasma adhering to the surface of melanoma cells as an initial step of colonization (indicated by arrows) (Rottem et al., 2012)

The above table is not exhaustive, and the methods employed are dependent upon the procedures used for the particular objective. The mycoplasmologists from The International Organization for Mycoplasmology (IOM) have developed two methods for the recognition of mycoplasma in cell-cultures – 'direct' and 'indirect'. Generally, the direct method refers to the standard approach of microbiological colony growing on agar which is aimed at detecting viable mycoplasma cells, while the indirect approach refers to processes and methods used to measure the gene products of mycoplasma such as proteins or enzymes, or to measure the metabolic and other properties of the affected cells (Drexler *et al.,* 2000).

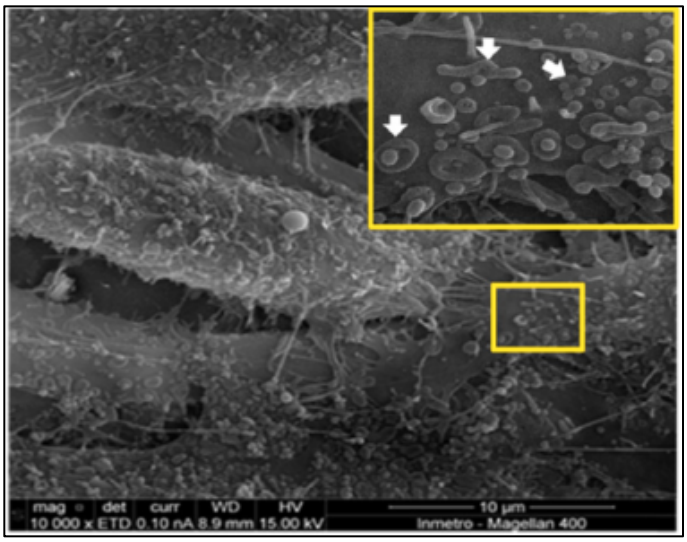

Figure 2.5. Scanning electron micrographs (SEM) of HGE-3 cells with mycoplasma contamination. The yellow box showing a magnification of individual mycoplasmas adhering into the surface of the HEG-3 cells (highlighted by arrows) (Falagan et al., 2015)

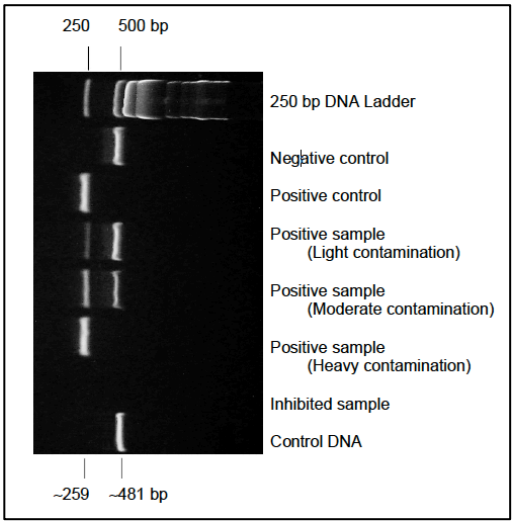

Figure 2.6. Mycoplasma detection using PCR detection method. The PCR includes amplification with specific primer set of the highly conserved 16S rRNA coding region in the mycoplasma genome. PCR product loaded into 2% agarose gel. Negative samples represent a single 500bp band while positive samples represent either single 250bp or double 500bp and 250bp bands. (SIGMA, LookOut Mycoplasma PCR detection protocol)

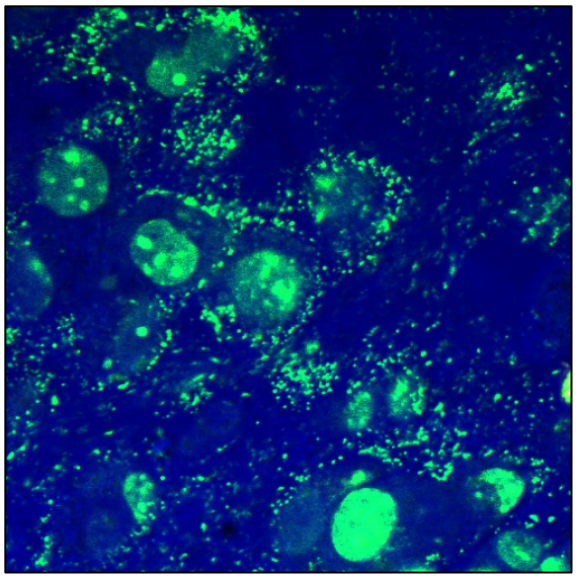

Figure 2.7. Mycoplasma contaminated cell culture stained with a fluorescent DNA stain. Mycoplasma appears on the cell surface as a filamentous or particulate pattern of bright fluorescence indicating mycoplasma DNA (Rottem et al., 2012)

However, many of these methods are exorbitant to use, because they are complex, difficult to implement and interpret, expensive and time consuming. Thus, there is a critical need of sensitive, specific, rapid, cost effective, efficient and easy to use and interpret methods to detect presence of mycoplasma (Uphoff *et al.,* 1992 & 2002; Drexler *et al.,* 2000 & 2002).

Knowing the variability of Mycoplasma detection methods, single detection method should not be relied upon and any results obtained should be confirmed by a second and separate method. However, it could be possible to use the same method,

with identical resource, in disperse locations, in order to check for the concordance of the results.

Currently, cell culture in broth and/or agar media such as Friis, PH-Hayflick, SP4 and DM-1 (Hayflick *et al.,* 1965; Uphoff *et al.,* 1992; Tully *et al.,* 1996) and PCR assays based on the 16S RNA sequence regions and 16S-23S intragenic regions of mycoplasma species have shown to be very reliable to detect mycoplasma (Drexler *et al.,* 2002; Razin *et al.,* 1994; Rawadi *et al.,* 1995; Harasawa *et al.,* 1999; Uphoff *et al.,* 2005; Timenetsky *et al.,* 2006).

2.8 Elimination And Eradication Of Mycoplasma In Cell Cultures

According to the International Organization for Standardization (ISO), all the cell cultures used either for studying the consequences of drugs and toxic complexes, mutagenesis and carcinogenesis, drug inspection and progress, and manufacturing of biological compounds (e.g., vaccines, therapeutic proteins) must be mycoplasma-free. Additionally, prestigious scientific journals such as Nature Biotechnology, and others, are also requires an author's statement of the use of mycoplasma-free and certified cells (UKCCCR, 2000; Nature Biotechnology Editorial, 2013). However, recent studies published in Nature, Cell, Genome Research, PNAS, and Genes and Development (top peer-reviewed journals) have reported high numbers of mycoplasma contamination (Olarerin George and Hogenesch, 2015)

It has been well established that in cell-culture, mycoplasma-free cell should be stock for routine laboratory work (McGarrity *et al.,* 1985; Uphoff *et al.,* 1992 &

2005). But where mycoplasma free cells are not freely available, or the cells are irreplaceable or valuable, then mycoplasma should be eradicate.

So far several techniques such as antibiotic treatment, co-cultivation of infected cells with macrophages, exposure to selectively toxic detergents, in vivo passage of constant cell lines through nude mice, induction of chromosomal damage using visible light and 5-bromouracile and use of anti-mycoplasma sera have been developed (Schmidt *et al.,* 1984, McGarrity *et al.,* 1985; Schmitt *et al.,* 1988; Drexler *et al.,* 2000 & 2002; Uphoff *et al.,* 2005). However, these techniques can be changeable, ineffectual, impractical, cannot be useful to every condition, time consuming, expensive and can cause changes to the characteristics and properties of the cells (McGarrity *et al.,* 1985; Schmitt *et al.,* 1988; Uphoff *et al.,* 1992; Drexler *et al.,* 2002).

The antibiotics remain the most dependable and effective approach for treating cells and cell-cultures infected with mycoplasma (Schmidt *et al.,* 1984). Amongst others, various Antibiotics are available for example; tetracycline, kanamycin, linomycin, erythromycin and tylosine which were used in various combinations and procedures for the elimination plus eradication of Mycoplasma. However, that resistance to several of the Antibiotics has been observed. Tiamutin and Minocyclin Antibiotics were noticed to be very efficient when used continuously for a number of weeks and that they did not produce any unwanted effects. It was then possible to clone the cells in order to produce an effective Mycoplasma free cell line (Schmidt *et*

al., 1984). Optimal results can also be achieved with the use of Ciprofloxacin and other Quinolones Antibiotics (Schmitt *et al.,* 1988, Uphoff *et al.,* 1992).

Tetracyclines, macrolides and quinolones were found to be the highest effectual Antibiotics against Mycoplasma. However, if applied in low concentrations they will have little or no effect but the possibility of the Mycoplasma cells and cell cultures developing a resistance to them will remain low. According to Uphoff, the most effective and therefore the Antibiotics of choice are Tiamutin, Minocycline, Quinolones and Mycroplasmal Removal Agent (MRA). Macrolide Tiamulin (BM-cyclin 1) and Tetracycline Minocycline (BM-cyclin 2) which bind to the 30S and the 50S ribosomal units, found to be the reason of inhibition of protein synthesis, while the inhibition of bacterial DNA replication (gyrase) is caused by quinolones and MRA (Uphoff *et al.,* 2005).

It has been reported that 4 weeks of time is essential for antibiotic treatment to be effective and to declare the mycoplasma free cells, treated cells should be monitor up to 6 weeks using the sensitive detection methods (Schmidt *et al.,* 1984, Schmitt *et al.,* 1988, Uphoff *et al.,* 1992 & 2005; Drexler *et al.,* 2000 & 2002).

CHAPTER 3. MATERIALS AND METHODS

3.1 MATERIALS AND REAGENTS

Table 3.1-5 contains a list of all the materials and their resources used in the research.

Table 3.1. LIST OF REAGENTS

Item	Company	Part Number
Agencourt AMPure XP	Beckman Coulter Genomics	A63881
Antibiotic Antimycotic Solution 100ml	SIGMA	A5955-100ML
Bionexus, Inc All Purpose Hi-Lo DNA Marker	Bionexus	BN2050
Chloroform: Isoamyl Alcohol 24:1	AMRESCO	X205-950ml
Dimethyl Sulphoxide DMSO	SIGMA	D2438
Disruptor beads	Electron Microscopy Sciences	72408-05
DMEM, high glucose, pyruvate	Gibco	11995073
Fetal Bovine Serum	SIGMA	F7524-500ml
Gel Loading Dye Blue (6X)	BioLabs	B7021S
PBS - Phosphate-Buffered Saline pH 7.4	Gibco	10010049
RNaseZap	Ambion	9782
TC10 Trypan Blue Dye	BIO-RAD	1450013
Trizol Reagent RNA	thermofisher	15596-026
Trypsin (.25%) EDTA (.1%) 500ml	SIGMA	59429C-500ML
VECTASHIELD®Mounting Medium with DAPI	VECTOR Laboratories	H-1200

Table 3.2. LIST OF KITS

Item	Company	Part Number
Agilent DNA 1000 Kit	Agilent	5067-1504
Agilent RNA 6000 Nano Kit	Agilent	5067-1511
BM-Cyclin	Roche	10799050001
HiSeq 3000/4000 PE Cluster Kit cBot (Box 1 of 2)	illumina	15067099
HiSeq 3000/4000 PE Cluster Kit cBot (Box 2 of 2)	illumina	15067100
HiSeq 3000/4000 SBS Kit 300 cycles (Box 1 of 2)	illumina	15067104
HiSeq 3000/4000 SBS Kit 300 cycles (Box 2 of 2)	illumina	15067105
LOOKOUT MYCOPLASMA PCR DETECTION KIT, LO	SIGMA	MP0035-1KT
Nextflex Rapid RNA-Sek kit	Bioo Scientific	5138-02
NEXTflex™ RNA-Seq Barcodes	Bioo Scientific	512912
TruSeq Multiplex Sequencing Primer	illumina	15017557
JUMPSTART TAQ DNA POLYMERASE, JUMPSTART	SIGMA	D9307-50UN

Table 3.3. LIST OF INSTRUMENTS

Item	Company	Part Number
2100 Bioanalyzer Laptop Bundle	Agilent	G2943CA
cBot	illumina	6001192
Confocal LSM 710	Carl ZEIZZ	LSM 710
FlashGel System	Lonza	57067
HiSeq 4000 Systems	illumina	sy-401-4001
TC20 automated cell counter	BIO-RAD	1450102

Table 3.4. LIST OF LAB SUPPLIES

Item	Company	Part Number
Cell culture flask vented, surface 75 cm2	BD FALCON	353136
Fisherbrand™ Disposable Borosilicate Glass Pasteur Pipets	FisherBrand	13-678-20D
FlashGel DNA Cassette 2.2% Agarose	Lonza	57031
Glass Bottom Microwell Dishes	MatTek	P35GC-0-10-C
Nalgene CN Filter Units 500 mL	Thermo Scientific	450-0020
NALGENE Cryo 1°C freezing container	NALGENE	5100-0001
Serological Pipette 10ml - 1/10ml, individual wrap case of 200	BD FALCON	357551
TC10 Counting Slides, box of 30 slides	BIO-RAD	1450011
TipOne® 101-1000 µl Filter Pipet Tips in Sterilized Racks, 10 racks of 96 tips (960 tips)	USA SCIENTIFIC	1126-7810
TipOne®1-200 µl Natural Pipet Tips in Sterilized Racks, 10 racks of 96 tips (960 tips)	USA SCIENTIFIC	1111-0810

Table 3.5. LIST OF LICENSED SOFTWARE

Item	Company
Ingenuity Pathway Analysis IPA	QIAGEN
PartekFlow	Partek

3.2 STUDY DESIGN

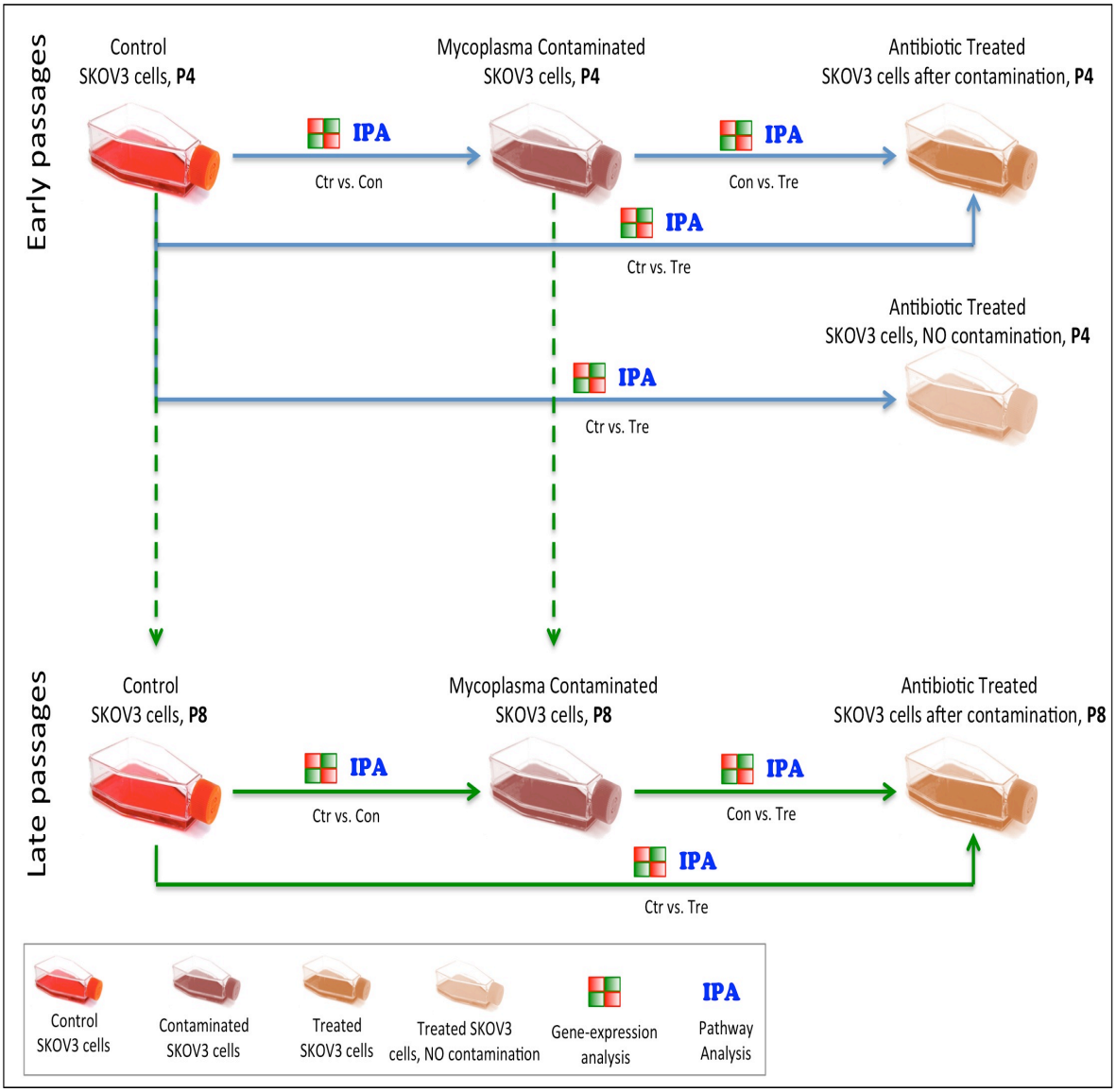

Figure 3.1. Cell-culture work's study design. Early passages represent 4 passages (P4) and late passages represent 8 passages (P8). The workflow illustrates time point of cell harvesting, gene-expression analysis and pathway analysis.

3.3 METHODS

3.3.1 ETHICAL APPROVAL

This study dose not includes any human/tissue samples and their related data, ethical approval is not required.

3.3.2 SAFETY

For safety purposes, laboratory safety registration documentation was obtained from Environmental Health and Safety department at WCM-Q (Appendix A). The documentation is certifying that the Genomics Core lab is safe to operate and the all research team members have received the required training to conduct research safely and in accordance with applicable institutional laws, regulations, and best practices.

3.3.3 SURVEY

To investigate the incidence of mycoplasma contamination in cell cultures and the most common methods used for mycoplasma detection and elimination in research laboratories in Qatar, a survey was developed using a web base Survey (https://www.surveymonkey.com). The survey included 9 multiple-choice questions (Appendix B). A link of the survey was sent to several research institutes in Qatar (https://www.surveymonkey.com/r/DV9BP69) and collected data were analyzed using SurveyMonkey web tools. The survey is anonymous, confidential and dose not implicates any risks to participant's work.

3.3.4 CELL CULTURE

SKOV3 human ovarian adenocarcinoma cell line (ATCC® HTB77) was purchased from (ATCC, USA). Cells handling and sub-culturing were performed according to manufacturer's protocol. Briefly, SKOV3 cells were maintained in high-glucose, Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% Fetal Bovine Serum (SIGMA®, USA) and 1% antibiotic antimitotic solution (SIGMA, USA). Prepared medium were filtered with Nalgene® vacuum filtration system. Cells were grown in a 75 cm2 culture flask at 37°C in 5% CO2. Medium was changed every 2–3 days. Upon achieving 70–80% confluence, cell were passaged with sub-cultivation ratio of 1:3.

3.3.5 CONTAMINATING CELL WITH MYCOPLASMA

SKOV3 cells were infected with mycoplasma using 10 µl of mycoplasma-contaminated medium (supernatant). This supernatant was obtained from the same, previously contaminated, cell line (SKOV3). The presence of mycoplasma in the supernatant was confirmed using Lookout® mycoplasma polymerase chain reaction (PCR) detection kit (SIGMA, USA).

3.3.6 CELL COUNT

Prior to each sub-culturing, number of growing cells was counted using TC10 Trypan blue dye/ TC20 automated cell counter (Bio-Rad).

3.3.7 DETECTION OF MYCOPLASMA

To determine mycoplasma contamination before harvesting and each passage, PCR assay and DAPI staining were used.

Lookout mycoplasma PCR detection kit was used according to the manufacturer's instructions (SIGMA, USA). The kit utilizes PCR technology using primers that are highly specific and conserved 16S rRNA coding region in mycoplasma genome. Before each sub-culturing of SKOV3 cells, 100 µl of supernatant were transferred to new tube, heated at 95°C for 5 minutes and incubated with PCR mix for 40 cycles (94°C for 30 seconds/ 55°C for 30 seconds / 72°C for 40 seconds) . A negative control (nuclease free water) and positive control (provided with the kit) were included in each reaction. PCR products were visualized on Fast Electrophoresis Lonza's FlashGel™ System (Lonza, USA) along with 100 bp DNA Marker (Bionexus, USA).

DAPI, (4',6-diamidino-2-phenylindole) a fluorescent dye that binds specifically to DNA, staining was performed for confocal microscopy imaging. Briefly, $2x10^4$ SKOV3 cells were grown in 2 mL of fresh medium in to a glass bottom microwell dishes until 70-80% confluence. Cells were briefly rinsed with 2 mL PBS. For fixation, cells were incubated with 500 µl of cold methanol at -20°C for 10 minutes. After washing with 2 mL of PBS, cells were stained by adding one to two drops (150 µl) of Vectashield Mounting Medium with DAPI stain (VECTOR Laboratories, USA). Cells were examined under confocal LSM 710 microscopy (Carl Zeiss, USA).

3.3.8 ELIMINATION OF MYCOPLASMA

BM- Cyclin (Roche, Germany), were used to eliminate mycoplasma contamination following manufacturer's protocol. Treatment with BM-cyclin included three cycles of antibiotic curse: BM-cyclin I (pleuromutilin derivative antibiotic) for 3 days followed by BM-cyclin II (tetracycline derivative antibiotic) for 4 days. To evaluate the efficiency of BM-Cyclin treatment, Lookout mycoplasma PCR detection kit was used.

3.3.9 RNA ISOLATION

Total RNA was extracted from $2x10^4$ SKOV3 cells with TRIZOL reagent (Thermo Fisher, USA). Briefly, cells were collected and washed twice with 1 mL of phosphate-buffered saline (PBS) (GIBCO®, USA) and distilled water. Cells were then lysed by vortexing with glass disruptor beads (Electron Microscopy Sciences, USA). For cell homogenization, 500 µl of Trizol was added, vortexed for 3 minutes and incubated at room temperature for 5 minutes. Following incubation, 140 µl of chloroform (AMRESCO, USA) was mixed and centrifuged for 15 minutes at 12000xg. After centrifugation, the aqueous top layer was transferred to new eppendorf tube and 250 µl of 100% isopropanol was added to precipitate RNA. RNA pellet was washed with 250 µl of 70% ethanol, and eluted with 50 µl of RNase free water. To determine RNA concentration and quality, Bioanalyze Agilent RNA 6000 Nano Kit (Agilent, USA) was used.

3.3.10 RNA-SEQ LIBRARY CONSTRUCTION

Following RNA isolation, 100 ng of total RNA was used to generate the RNA-seq libraries using NEXTflex Rapid RNA-Seq Kit (Bioo Scientific, USA) according to the manufacturer's instructions. Briefly, RNA was fragmented using a cationic buffer. Fragmented RNA undergoes first and second strand synthesis, followed by end-repair, adenylation, adapter ligation (NEXTflex™ RNA-Seq Barcodes) and PCR purification. Amplified DNA library was purified with Agencourt AMPure XP Bead (Beckman Coulter Genomics, USA). The final libraries were quantified using Agilent Bioanalyzer 1000 DNA assay (Agilent, USA) and 8 libraries per pool were pooled.

3.3.11 NEXT-GENERATION SEQUENCING (NGS)

Paired-end 150 bp sequencing was carried out on HiSeq 400 (Illumina. USA), according to the manufacturer's instructions. Clustering and amplification of constructed RNA-Seq libraries were conducted on cBot (illumine, USA) for 4 hours. The beaded flow cell was then loaded into the HiSeq 4000 and sequenced using HiSeq 3000/4000 SBS Kit 300 cycles (illumine, USA). Sequencing took around 72 hours.

3.3.12 DATA ANALYSIS

Collected raw data from HiSeq 4000 were analyzed using PartekFlow (Partek, USA) following the workflow demonstrated in figure 3.2.

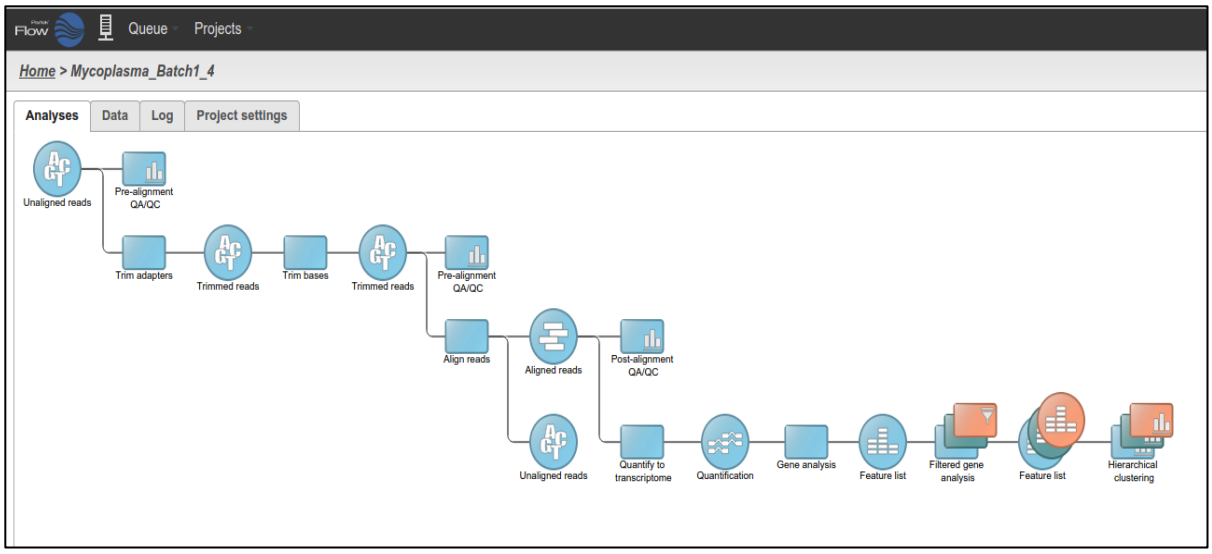

Figure 3.2. PartekFlow workflow for RNA-Seq analysis, showing multiple steps of quality checking, sequence trimming, alignment, gene analysis filtrations and heat-map clustering.

Samples were first assigned to three groups: control, contaminated and treated. Fastq files were checked for reads quality and then aligned to human reference (hg19) and mycoplasma reference using Bowtie2 aligner. To estimate transcript abundance of each condition, Partek's "optimization of the expectation-maximization algorithm" was used. Differentially expressed genes were detected using gene specific analysis (GSA) and filtered with the followings cutoffs: *P-value* = 0.01, fold-change = <-2, >2) and fouls discovery rate FDR = 0.01. Generated differentially expressed genes lists were imported to Ingenuity pathway analysis (IPA) (QIAGEN, UAS) for pathway analysis.

CHAPTER 4. RESULTS

4.1 Survey of the incidence of mycoplasma contamination in research laboratories in Qatar

Figures 4.1-8 showing responses from five research laboratories and institutes in Qatar [Anti-Doping Lab, Hamad Medical Corporation (HMC), Qatar Biomedical Research Institute (QBRI), Qatar Cardiovascular Research Center (QCRC), Qatar Science and Technology Park (QSTP), Qatar University (QU) and Weill Cornell Medicine-Qatar (WCM-Q)]. The incidence of mycoplasma contamination reached to 42.9% in Qatar (Figure 4.3). The survey addresses the most commonly used cells on cell culture work, ESC Embryonic Stem Cell followed by SKOV3 human ovarian adenocarcinoma cell and HEK 293 Human Embryonic Kidney (Figure 4.2). It also addressing the most preferable methods used for mycoplasma detection (LookOut® mycoplasma PCR Detection Kit from Sigma followed by DAPI Staining) and elimination (BM-Cyclin from Roche followed by LookOut® mycoplasma Elimination Kit from Sigma) in Qatar (Figure 4.4 and 4.6).

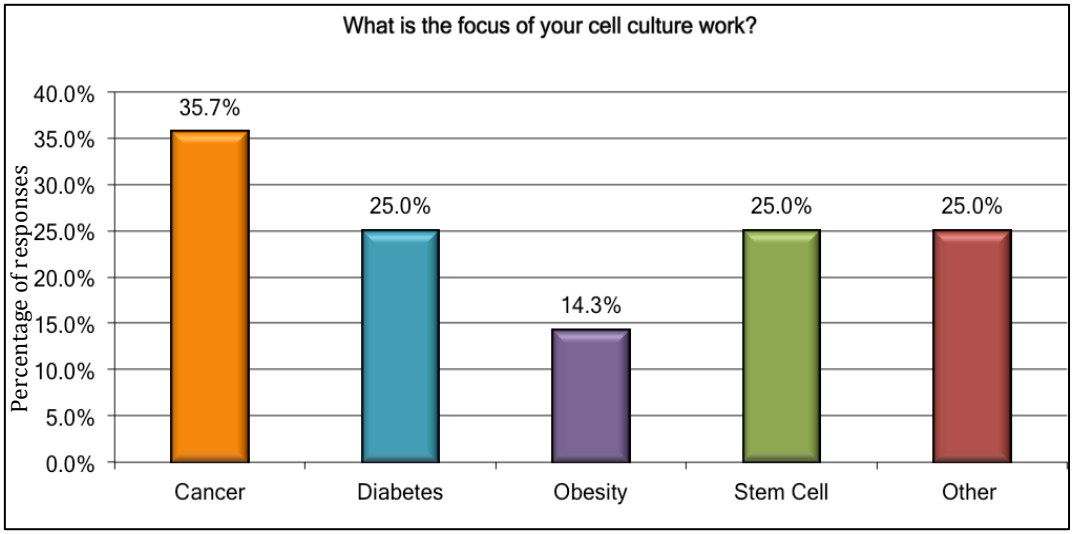

Figure 4.1. Most common fields of cell-culture work at Qatar, other: (Cell cycle, Cell signaling, Quality Control, Bacteriology, Plant tissue culture, Tissue engineering, and Calcium signaling)

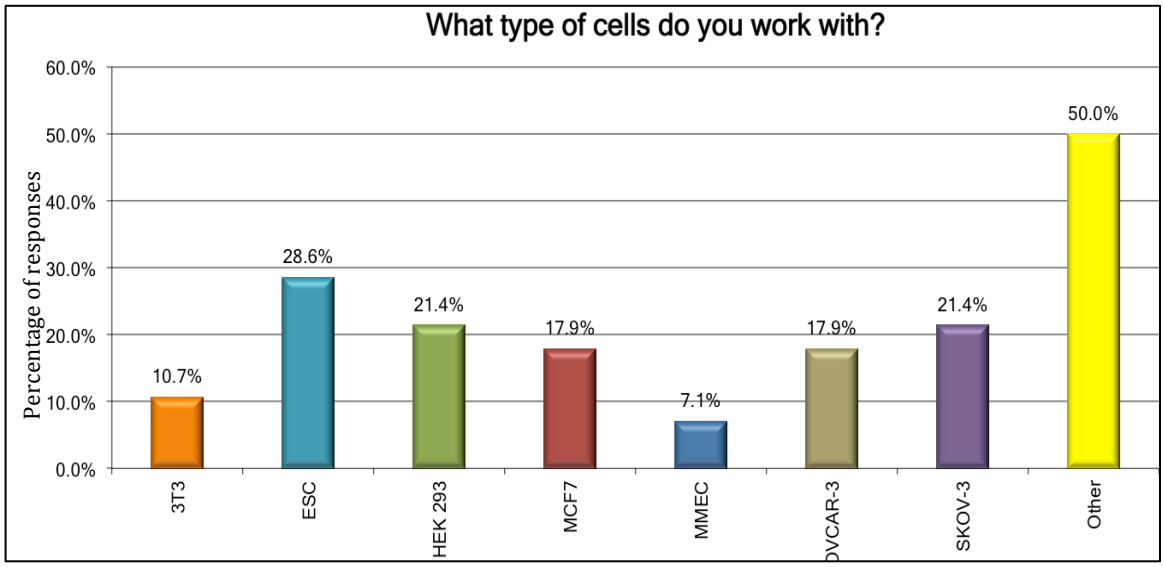

Figure 4.2. Most commonly used cell lines at Qatar, others: (Human pancreatic cell lines, (CHO) Chinese hamster ovary cells, breast cancer cell lines, HUVEC, adipocyte, Hela, CML, mesenchymal stem cells, S2B2).

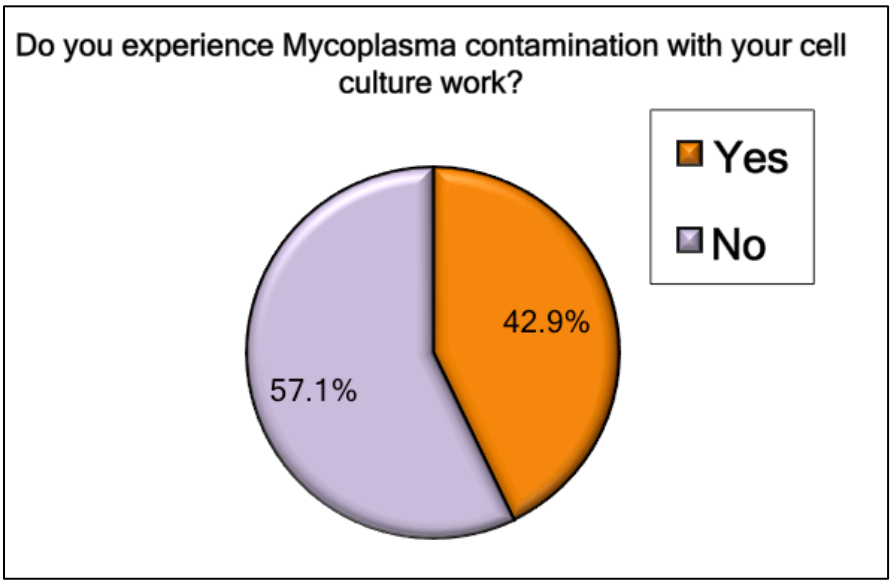

Figure 4.3. Frequency of mycoplsma contamination at Qatar

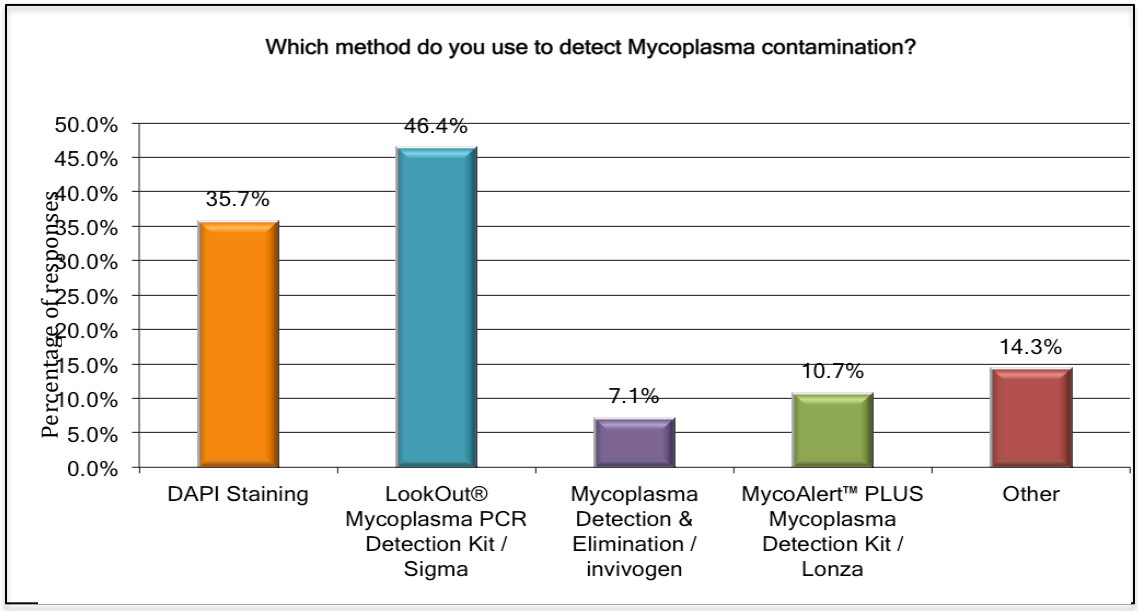

Figure 4.4. Most commonly used mycoplasma detection methods at Qatar, others:

(custom made primers and appearance)

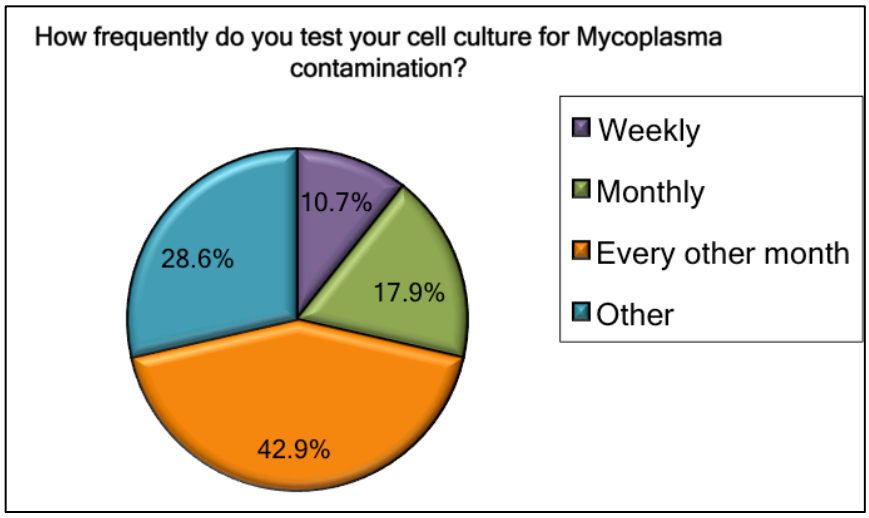

Figure 4.5. The frequency of testing for mycoplasma contamination at Qatar, other: **(**before having an important experiment, When needed, Randomly, Every 3 months, daily, Only if we suspect**)**

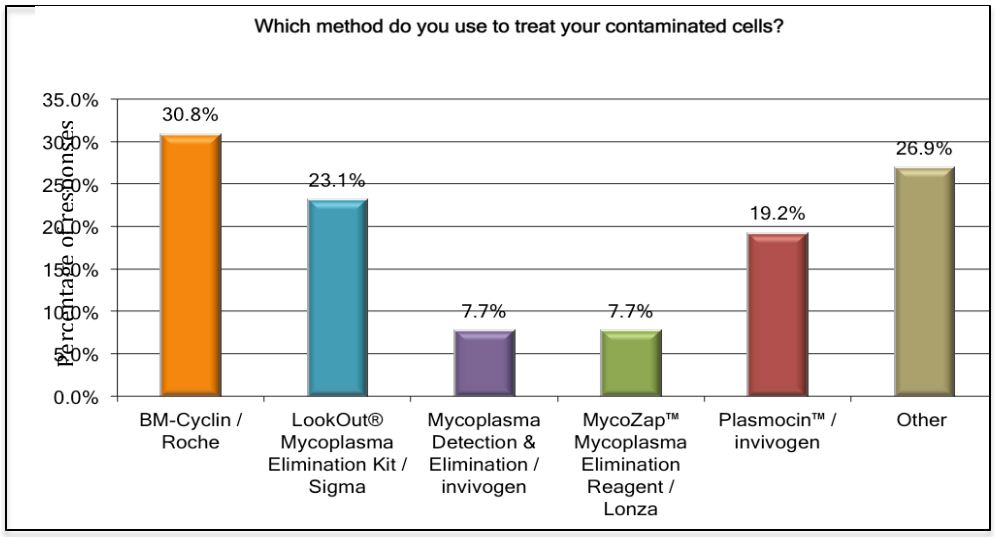

Figure 4.6. Most commonly used mycoplasma elimination methods in Qatar, others: (Discard contaminated cells and start new culture, antibiotics)

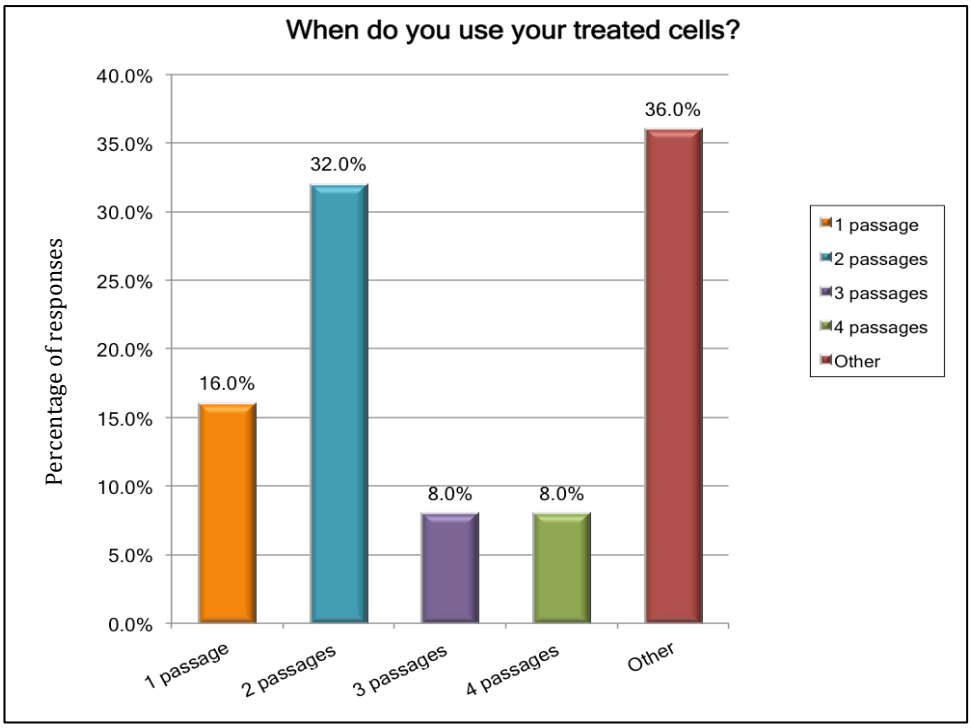

Figure 4.7. Number of passages allowed after treating cells with anti-mycoplasma in Qatar, others: (Once the BM cyclin treatment is done, 6 passages)

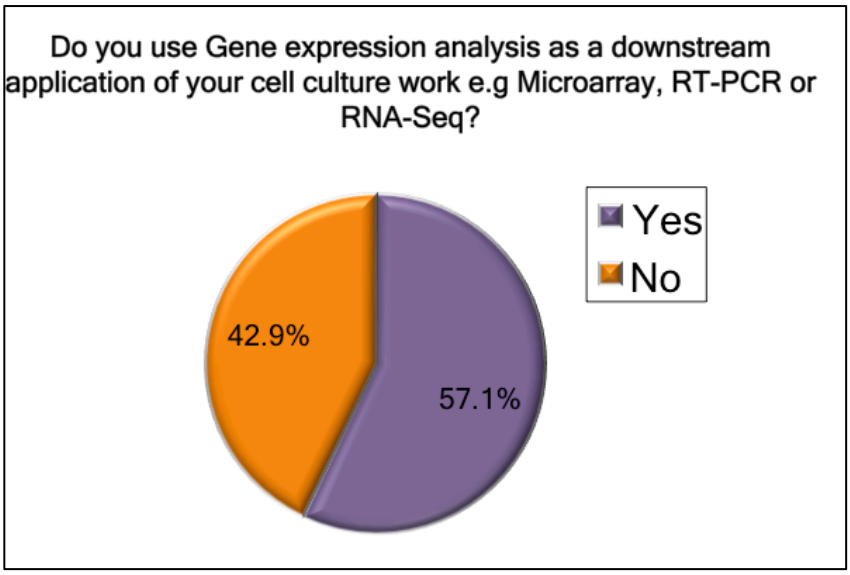

Figure 4.8. Frequency of using cell culture work for gene-expression studies

4.2 Mycoplasma contamination test using PCR method

Mycoplasma detection by PCR assay was used for three purposes: to confirm the absence of mycoplasma on control cells, to confirm the existence of mycoplasma on contaminated source (supernatant) and to examine mycoplasma contamination before sub-culturing. Agarose gel showed either single (250 bp) or two bands (250 bp and 500 bp) in positive control and positive sample, however, in negative control only single band appears at 500 bp.

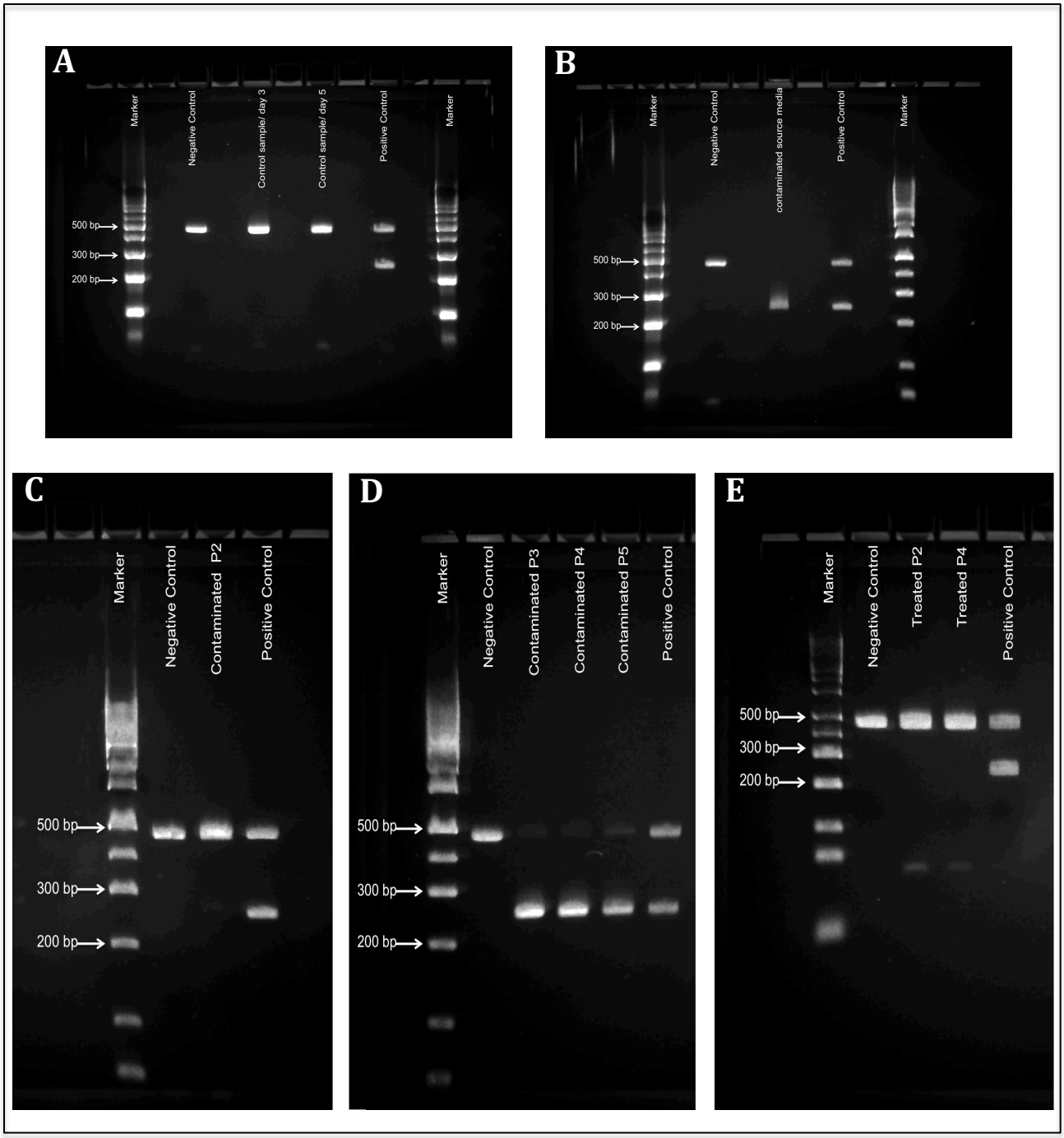

Figure 4.9. Gel electrophoresis of PCR product of mycoplasma PCR detection test. PCR loaded into 2.2% agarose cassette. **A)** control samples after 3 and 5 days of sub-culturing. **B)** contaminated supernatant. **C, D)** contaminated cells at 2 to 5 passages. **E)** passages 2 and 4 of antibiotic treated cells.

4.3 Effect of mycoplasma contamination on cell morphology

Figure 4.10. A & B shows the normal morphology (epithelial cell) of SKOV3 cells with normal distribution at the second passage. Contaminated SKOV3 cells with mycoplasma were still preserving both normal cell morphology and cell counts after the third passages. Figure 4.10.C, at the eighth subcultures, the effect of mycoplasma was showing as cells aggregation and irregular distribution Figure 4.10.D.

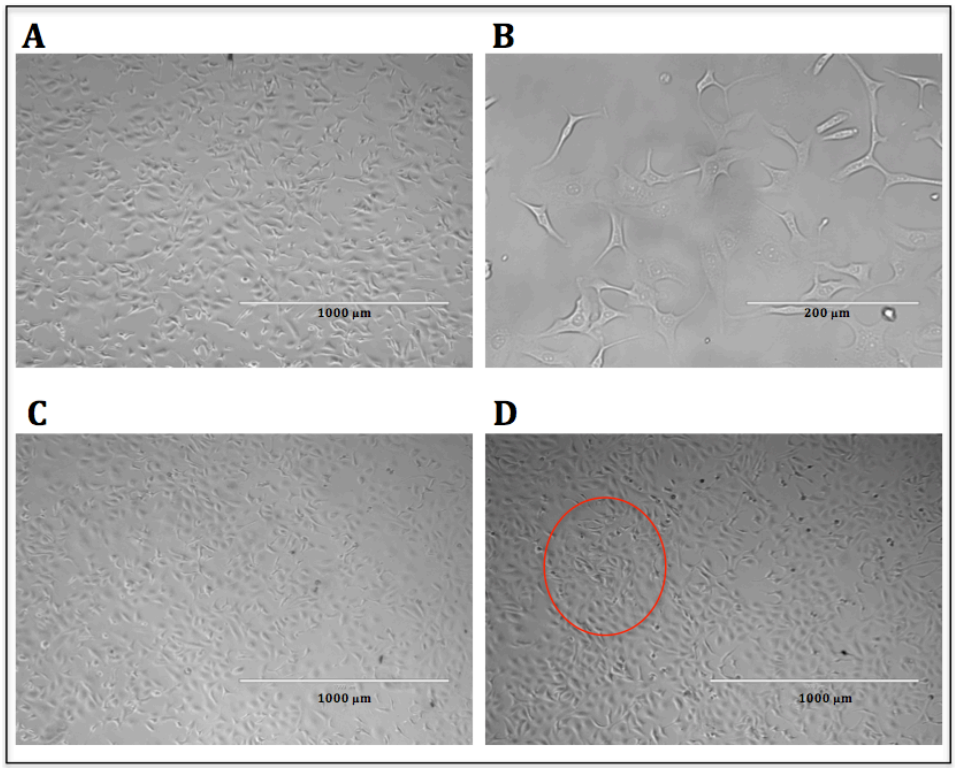

Figure 4.10. Effect of mycoplasma contamination on cell morphology. A, B) Microscopic image of the second subculture of control SKOV3 cells. **C, D)** Microscopic image of the third and eighth subculture of Contaminated SKOV3 cells with Mycoplasma. Red circle is pointing to cell aggregation.

4.4 Mycoplasma contamination test using DAPI staining

Figure 4.11 showing confocal microscopic imaging of control and mycoplasma contaminated SKOV3 cells after staining with DAPI stain. Contaminated cells were showing absences of mycoplasma in passage 2 to 8.

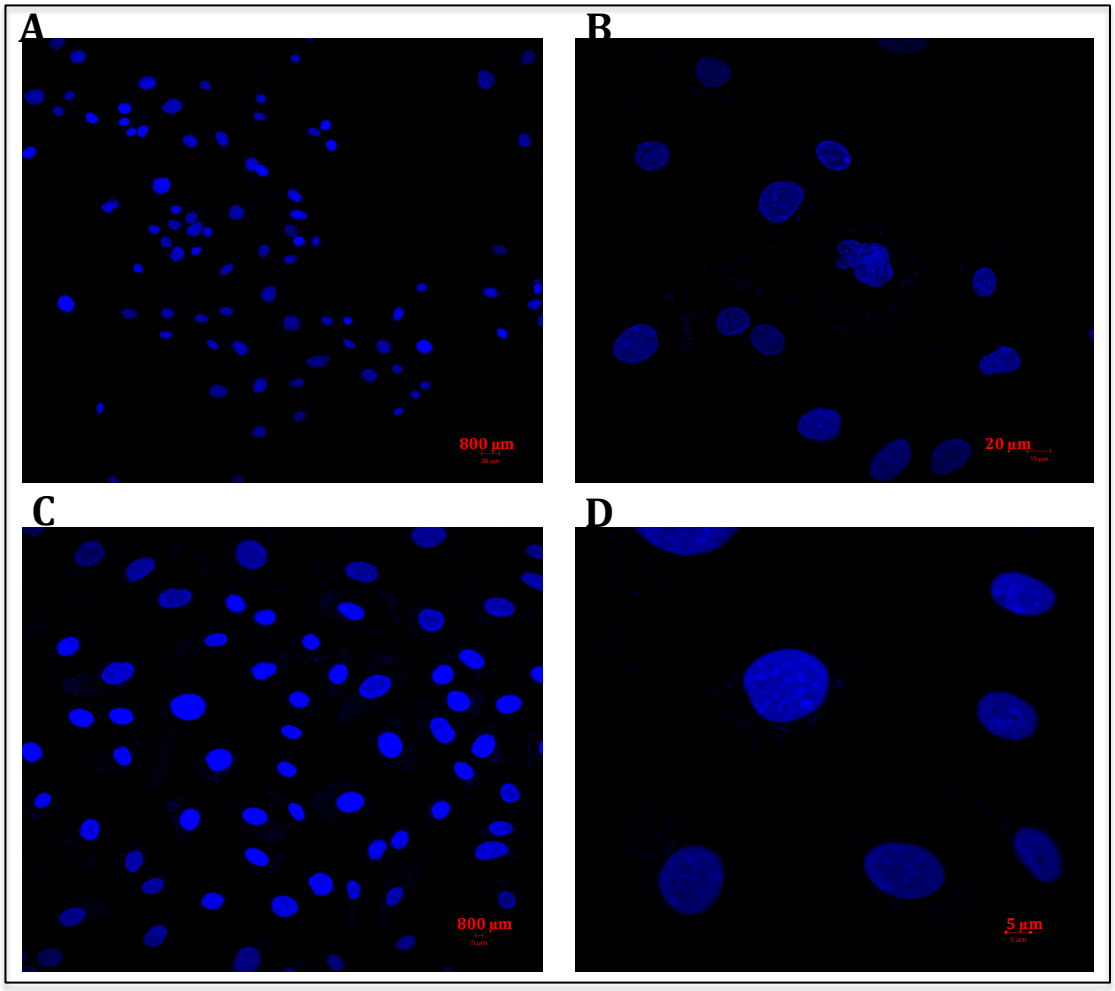

Figure 4.11. **Confocal microscopic imaging of control and mycoplasma contaminated SKOV3 cells after staining with DAPI stain. A, B)** Passage 3 of Mycoplasma contaminated SKOV3 cells. **C, D)** Passage 8 of Mycoplasma contaminated SKOV3 cells.

4.5 Effect of mycoplasma on gene-expression profile of control SKOV3 cells and the associated pathway analysis

Figure 4.12 A & B showing differentially expressed genes of contaminated SKOV3 cells in comparison to control cells and the top enriched biological networks that were affected by mycoplasma. In early passages of mycoplasma contamination, a total of 119 genes were significantly dis-regulated (94 genes down-regulated and 25 genes up-regulated). While in late passages of mycoplasma contamination, the number of significantly dis-regulated genes was increased to 924 genes (745 genes down-regulated and 179 genes up-regulated). All differentially expressed genes are clustered based on significant genes with a cutoff = *P*-value with FDR < 0.01 and Fold change $> 2 < -2$. Affected biological pathways of both early and late passages of contaminated cells were almost similar but with different enrichment scores. The most affected pathways are related to cell death and survival, cell to cell signaling, cell movements and immune cell trafficking (Figure 4.12 C & D).

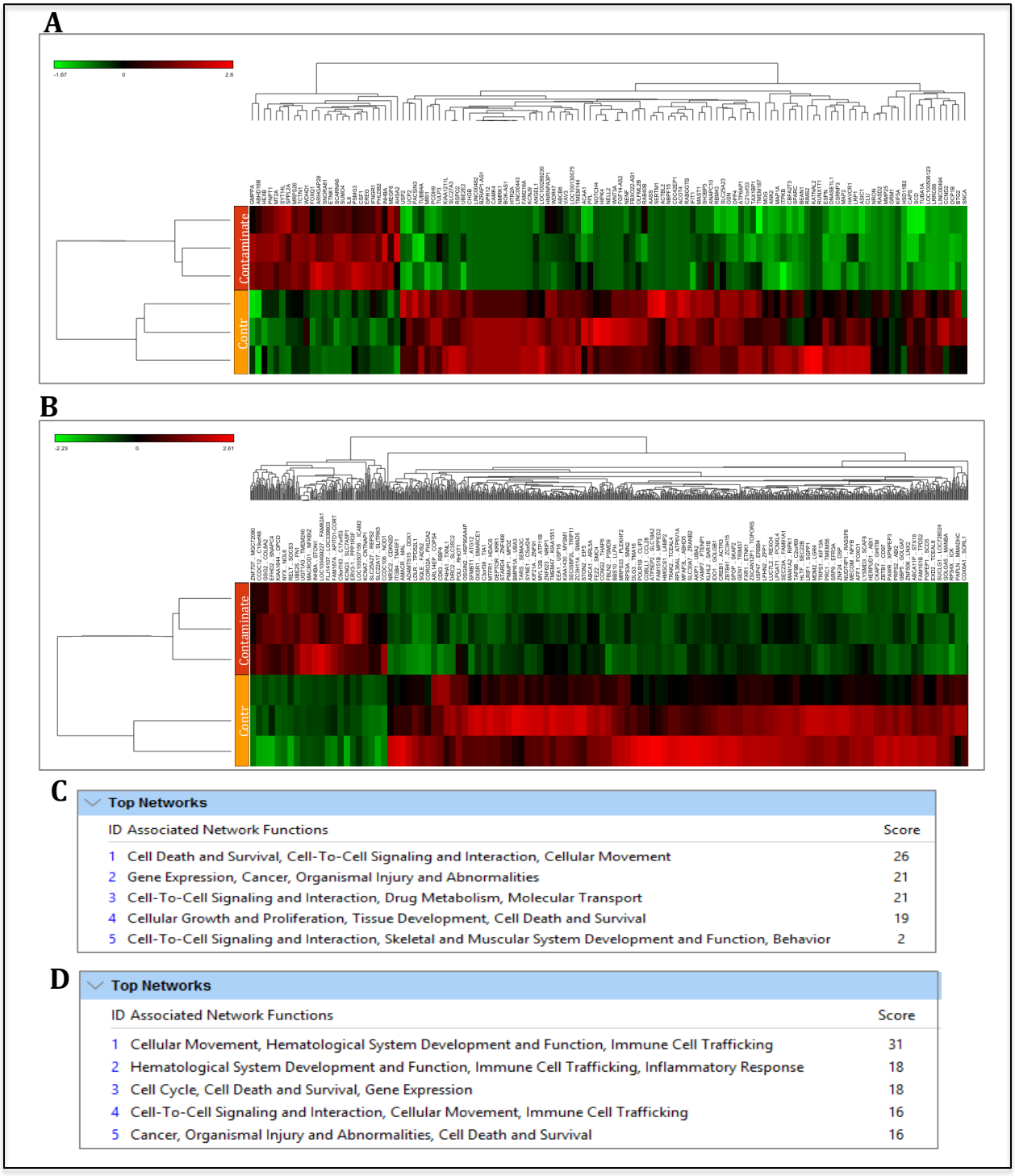

Figure 4.12. A) Gene-expression heat-map of contaminated SKOV3 cells vs. Control cells in early passages and **B)** late passages of contamination, (green = down regulated, red = up regulated) **C)** top enriched biological pathway affected by mycoplasma in early passages and **D)** late passages of contamination.

4.6 Effect of antibiotic treatment on gene-expression profile of contaminated SKOV3 cells and the associated pathway analysis

Before proceeding with gen-expression analysis of treated cells, cell morphology and mycoplasma PCR test where checked. After treatment, mycoplasma contamination was successfully removed and SKOV3 cells were representing normal epithelial morphology as shown on Figure 4.13 A & B. Figure 4.14-A & B showing differentially expressed genes in contaminated SKOV3 cells after antibiotic treatment in comparison to contaminated cells. In early passages of antibiotic treatment a total of 1586 genes were significantly dis-regulated (1559 genes down-regulated and 27 up-regulated). While in late passages of antibiotic treatment, the number of dis-regulated was deceased to 146 genes (100 genes down-regulated and 46 up-regulated). All differentially expressed genes are clustered based on significant genes with a cutoff = *P*-value with FDR < 0.01 and Fold change $> 2 < -2$.

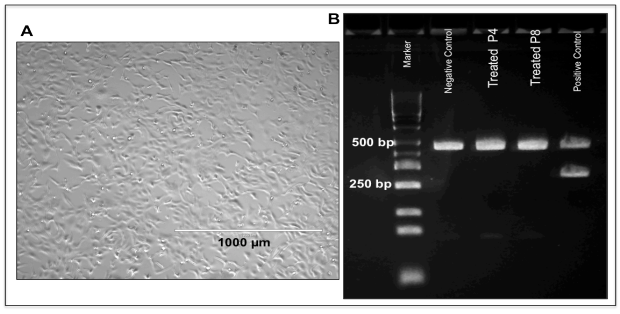

Figure 4.13. Effect of antibiotic treatment on SKOV3 cell morphology. A) SKOV3 cell after antibiotic treatment showing normal epithelial cell morphology after 8 passages. **B)** Gel electrophoresis of PCR product of mycoplasma PCR detection test of SKOV3 cells after antibiotic treatment, showing negative results. PCR loaded into 2.2% agarose cassette

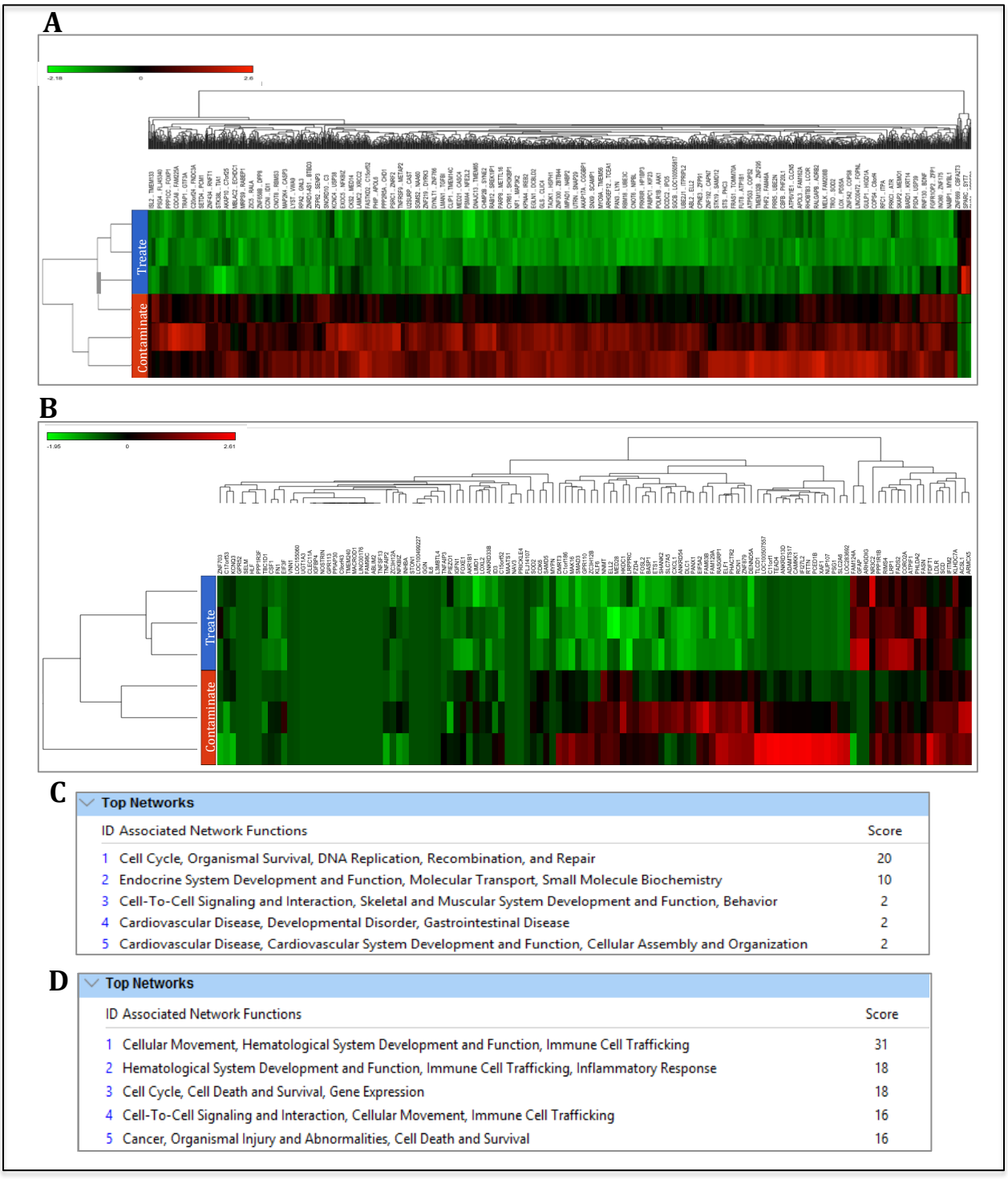

Figure 4.14. A) Gene-expression heat-map of antibiotic treated SKOV3 cells vs. mycoplasma contaminated cells in early passages and **B)** late passages (green = down regulated, red = up regulated)**, C)** top enriched biological pathway affected by antibiotic treatment in early passages and **D)** late passages of treatment.

4.7 Examine the continuation effect of mycoplasma on SKOV3 cells

To examine the continuation effect of mycoplasma on SKOV3 cells, differentially expressed genes of treated SKOV3 cells vs. control cells were identified first. In early passages of antibiotic treatment a total of 1586 genes were significantly dis-regulated (1225 genes down-regulated and 4 up-regulated). While in late passages of antibiotic treatment, a total of 793 genes were significantly dis-regulated (776 genes down-regulated and 17 up-regulated). All differentially expressed genes are clustered based on significant genes with a cutoff = *P*-value with FDR < 0.01 and Fold change $> 2 < -2$ (Figure. 4.15-A & B).

Then, list of affected genes before antibiotic treatment were overlapped with list of genes after antibiotic treatment. Overlapping of early passages showed that 66 genes (55 %) of differentially expressed genes in contaminated SKOV3 cells were disappeared after antibiotic treatment. While in late passages, 74 genes (62%) were disappeared after antibiotic treatment (Figure. 4.15-E & F).

Pathway analysis of affected genes by antibiotic treatment showed enrichment on carbohydrate metabolism pathway with enrichment score of 24. Dis-regulation of this pathway will effect the ATP production in fermentative mycoplasma by disrupting glycolytic pathway as discussed earlier.

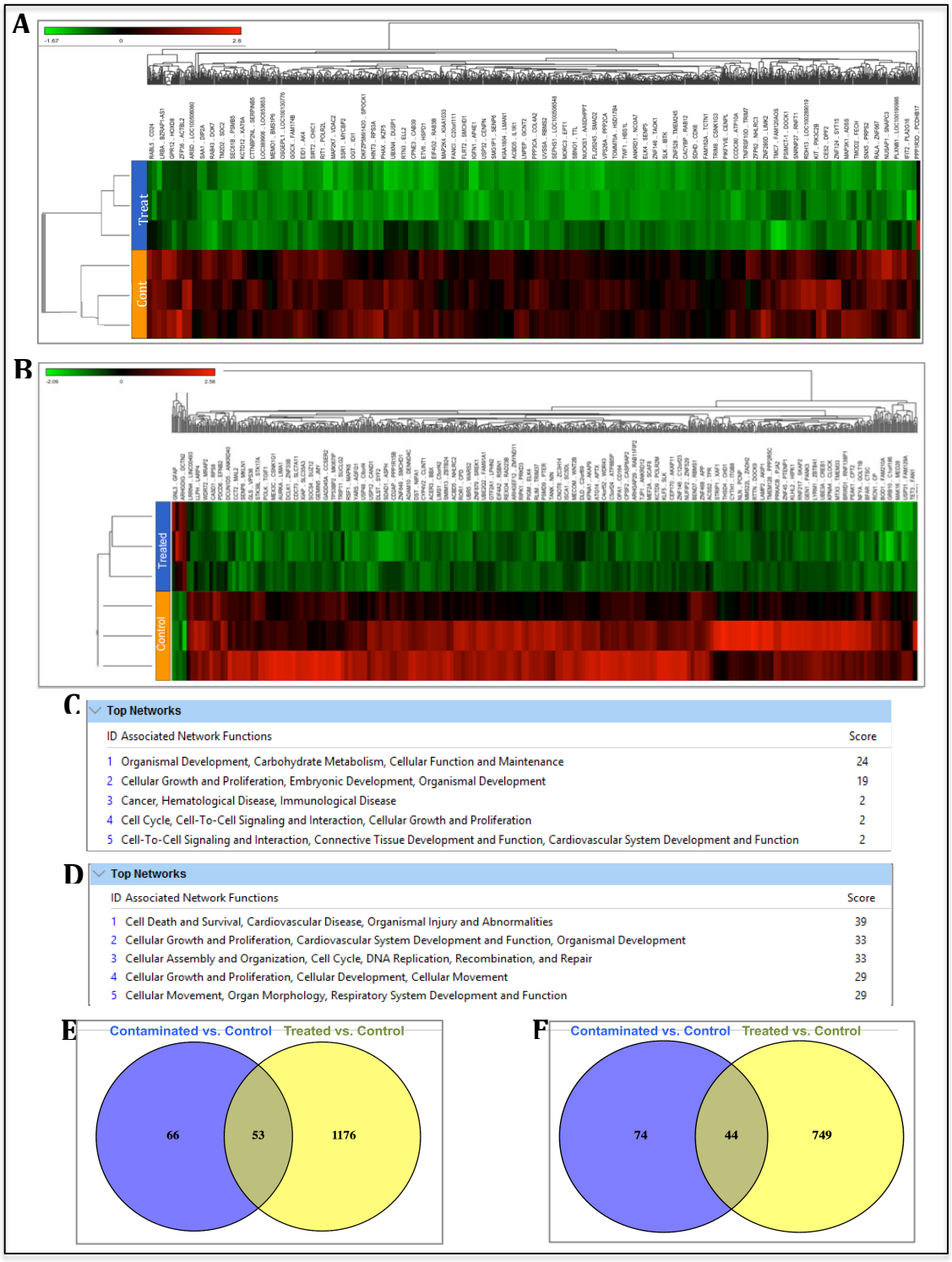

Figure 4.15. A) Gene-expression heat-map of treated SKOV3 cells vs. control cells in early passages and **B)** late passages (green = down regulated, red = up regulated)**, C)** top enriched biological pathway affected by antibiotic treatment in comparison to control cells in early passages and **D)** late passages, **E)** overlapping of differentially expressed genes of contaminated vs. control SKOV3 cells and treated vs. control SKOV3 cells in early passages and **F)** in late passages.

4.8 Validation of antibiotic treatment affects on control SKOV3 cells

In order to validate that antibiotics cause the increasing number of dis-regulated genes after treatment, control SKOV3 cells were treated with antibiotics directly without any previous contamination. Figure 4.16-A showing differentially expressed genes in treated SKOV3 cells with no previous contamination in comparison to control cells. Around 201 genes were significantly dis-regulated (94 genes down-regulated and 152 up-regulated). All genes are clustered based on significant genes with a cutoff = *P-value* with FDR < 0.01 and Fold change $> 2 < -2$ and their top enriched biological pathway analysis.

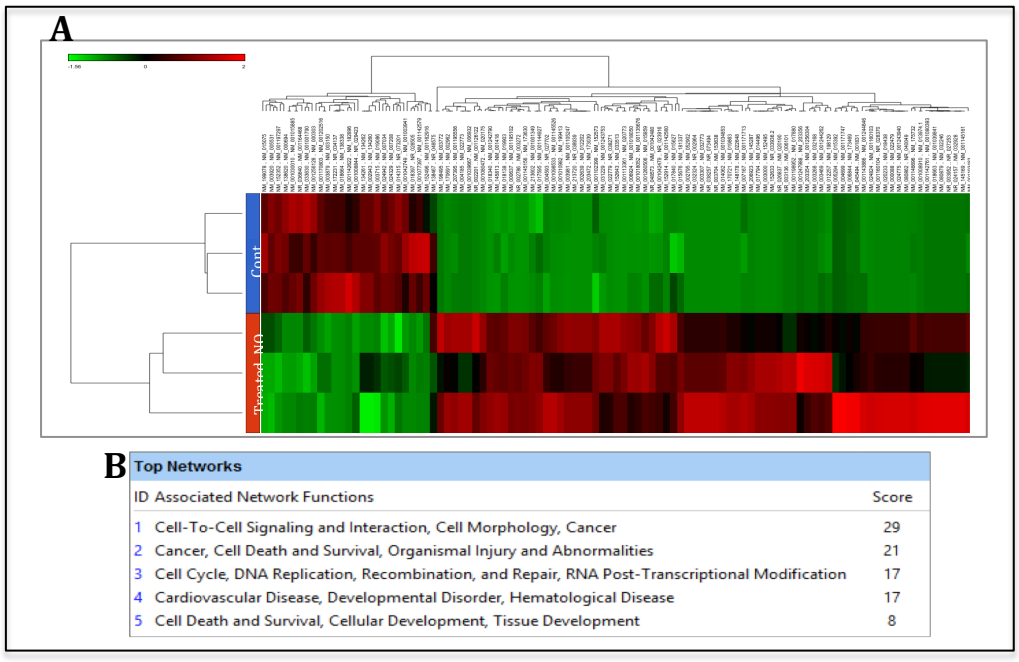

Figure 4.16. A) Gene-expression heat-map of treated SKOV3 cells with NO contamination vs. control cells, (green = down regulated, red = up regulated). **B)** Top enriched biological pathway that affected by antibiotic treatment

CHAPTER 5. DISCUSSION AND FUTURE DIRECTIONS

1.5 DISCUSSION

Cell-culture has become the most common biological model used in biology and medical research (Zalai et al., 2015). The use of cell-culture will continue to expand in the future due to restricted human and animals laws and protocols. Therefore, to insure the validity and significance of cell-culture experiments, it is necessary to control the right environment of cell-culture work and prevent any artificial changes, like unadjusted chemicals or nutrients and most importantly to prevent cell contamination.

Mycoplasma contamination considered one of the serious problems in cell-culture (Laleh et al., 2011). The effect of mycoplasma contamination in cell-culture is well reported and studied. These effects can range from visible effects, like cells physiology and metabolism alteration or invisible effects, like gene-expression alteration, which is more dangerous (McGarity et al., 1984 & 1985; Drexler et al., 2000 & 2002). To date, several methods have been developed for mycoplasma detection and elimination in cell-culture (Schmidt et al., 1984).

Antibiotics treatment of contaminated-cells showed the ability to eliminate the visible effect of mycoplasma contamination. In this study, besides identifying the effect of mycoplasma in gene-expression profile, we also evaluated the ability of antibiotics treatment in reversing these changes (invisible changes). The aim of this study is to answer the research question, whether mycoplasma contaminated cultured cells can be used or not.

The work of this study started with choosing suitable experimental variables like, cell lines, mycoplasma detection and elimination methods, number of cell passages and timings required. For this purpose, a survey was established, which investigate the incidence of mycoplasma contamination in cell-cultures and most frequently methods used for the detection and elimination of mycoplasma in research laboratories in Qatar. Based on the survey, the variables on table 5.1 were selected.

Table 5.1. Selected variables for cell-culture work

Cell Line:	SKOV3 cells
Mycoplasma detection method:	LookOut® Mycoplasma PCR Detection Kit from Sigma and DAPI Staining
Mycoplasma elimination method:	BM-Cyclin from Roche
Number of passages before using Antibiotic treated-cells:	4 passages

SKOV3 cell line was selected for this study although it was highlighted as the second most common used cell line after Embryonic Stem Cells (ESC) in Qatar. However, ESC needed a special precautions and treatments and were not available at the time of this study. Previous studies showed that PCR based assays are the most sensitive methods for mycoplasma detection (Drexler et al., 2002). In the present study LookOut® Mycoplasma PCR Detection Kit was the most common method to be used for mycoplasma detection in Qatar. The PCR based detection method was

able to detect mycoplasma contamination from the first passage of contaminated cells. Furthermore, the DAPI staining had shown lower sensitivity (Nikfarjam & Farzaneh 2012). In this study DAPI staining failed to detect mycoplasma contamination even after eight passages (56 days) of contamination. Nevertheless, we found that 35.7% of laboratories in Qatar using DAPI staining as the main method for mycoplasma detection. Along with regular microscopic examination, DAPI staining could also be used as a method to detect cell morphological changes caused by mycoplasma contamination. However, these changes are very slow and only can be detected at late stages of contamination. Similarly, we also found that cell morphological changes and cell aggregation presented after eight passages of contamination.

The effectives of BM Cyclin kit (includes Tiamulin and Minocycline antibiotics) in curing mycoplasma contamination were evident from the first round of treatment in this study. This result was expected since several comparative studies reported that BM Cyclin kit is the most effective method for mycoplasma elimination (Uphoff et al., 2005).

Our data survey on incidence of mycoplasma contamination in Qatar laboratories shows higher (42.9%) rates compared to the rest of the world (35%). Additionally, the survey also revealed that more than half of the research institutes in Qatar (57.1%) are using gene-expression analysis as a downstream application of their cell-culture work. Indeed, to prevent mycoplasma contamination, these high percentages should raise more considerations and precautions that should be followed

when dealing with cell-culture experiments in Qatar. Our findings suggest that routine mycoplasma testing of cultured cells should be performed in research laboratories in Qatar. The majority (28%) of research laboratories in Qatar do not regular testing their cell culture for mycoplasma contamination.

The experimental design of this study aimed to mimic the real situation of mycoplasma contamination in cell-culture work. Which mostly occurs from cross contamination with other contaminated-cells or medium that could be represented on contaminated consumables like tips or splashed drops on the cell-culture cabinets (Hay et al., 1989). In the present study, 10 µl of contaminated media (supernatant) from previously contaminated SKOV3 cells were used to contaminate our SKOV3 cell, despite the kind of mycoplasma species included on the supernatant. Supernatant of the same type of cell line were used to rule out the effect of any cell secretion (e.g. proteins, growth factors or cytokines) that differs form cell to cell type (Dowling & Clynes, 2011).

The RNA-Seq analysis showed that mycoplasma dis-regulate gene-expression profile of SKOV3 cells, which are in agreement with reports (Miller et al., 2003; Zhang et al., 2006; Hopfe et al., 2013). We identified 119 dis-regulated genes in early passages, while in late passages 924 genes were dis-regulated and highly significant (*P-value* with FDR < 0.01 and Fold change $> 2 < -2$). In addition, > 70% of these dis-regulated genes were down regulated. Pathway analysis of dis-regulated genes highlighted cell death and survival, cell-to-cell signaling and interaction and cell movement as top enriched biological pathways. Dis-regulation of these pathways

could explain the aggregation of contaminated-cells (Figure 4.10-D). Despite the high number of dis-regulated genes, we observed a slight un-noticeable changes on cell morphology represented as cell aggregation.

Antibiotic treatment of contaminated cells, was able to eliminate the contamination, as stated above, however, genes dis-regulation were irreversible. More interestingly, antibiotic treatment showed more dis-regulation on the gene-expression profile. Unlike the effect of mycoplasma, antibiotic treatment showed a shrinking pattern on the number of dis-regulated genes. In early passages, 1586 genes were dis-regulated, while in late passages the numbers of dis-regulated genes were shrinked to 146 genes and >60% were down regulated.

Our data suggests that the more passages after antibiotic treatment will give more reduction of antibiotic effect on the gene-expression profile. Though, further studies should be carried out to validate and evaluate the possibility of reversing all genes dis-regulation. The survey demonstrates that 32% of scientist in Qatar used the treated-cells only after two passages. However, based on our results, two passages are not enough to make sure that treated cell are ready to be used.

Overlapping dis-regulated genes list SKOV3 cells before and after treatment, showed that some of dis-regulated genes (55% in early passages and 62% in late passages) were cured. While some genes were remain dis-regulated after treatment and other genes were dis-regulated uniquely after antibiotic treatment. These results led to the following concern, does the newly dis-regulated genes after

antibiotic treatment are due to the persistent effect of mycoplasma or due to the antibiotic effect!

Thus, to study the effect of antibiotics on cells, we used antibiotic treated SKOV3 control cells without prior contamination and observed that the genes were dis-regulated due to the effect of antibiotic.

Findings of this study concludes that contaminated cells with mycoplasma should not be further used or processed, specially for gene-expressions studies, due to the significant gene dis-regulation as consequence of either mycoplasma or antibiotics effects. Providentially, 6% of scientist in Qatar showed a habit of discording cells once they were contaminated. Nonetheless, these findings were obtained by studying one specific cell line (SKOV3). Hence, to generalize the effect of mycoplasma contamination and anti-mycoplasma treatment on gene-expression profile, other cell lines and antibiotics should be tested.

5.2 FUTURE DIRECTION:

- In order to validate and generalize the effect of mycoplasma contamination and anti-mycoplasma treatment on gene-expression profile, other cell lines and antibiotics will be tested in the future.
- To understand the mechanism of witch mycoplasma dis-regulates gene-expression of cultured cells, protein profile of contaminated cells will be studied using mass spectrometry (MS).

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APPENDIX A: Institutional Biosafety Committee Approval (WCM-Q)

Weill Cornell
Medicine-Qatar

Date: April 17, 2016

Re: Iman K. Al-Azwani
Research Specialist IV
Genomics core lab
Weill Cornell Medicine - Qatar

To Whom It May Concern:

This letter is to certify that the Genomics Core Laboratory at Weill Cornell Medicine – Qatar where Ms. Iman K. Al-Azwani works is safe to operate and research team members have received the required training to conduct research safely and in accordance with applicable institutional laws, regulations, and best practices.

Please let us know if you have questions or need additional information.

Sincerely,

Thomas L Doyle

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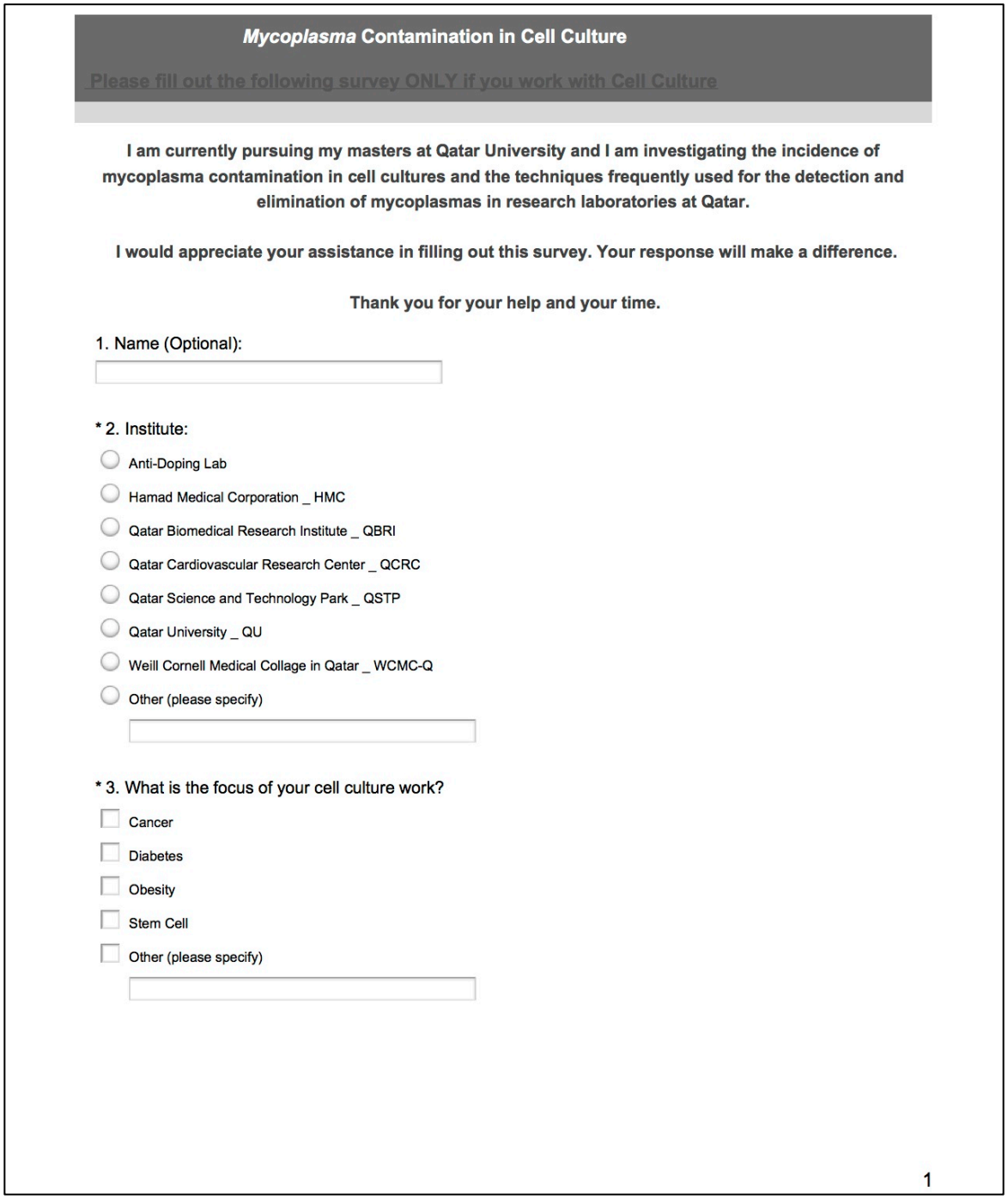
APPENDIX B: Survey of incidence of Mycoplasma contamination in cell cultures and the techniques frequently used for the detection and elimination of Mycoplasma in research laboratories in Qatar



***Mycoplasma* Contamination in Cell Culture**

Please fill out the following survey ONLY if you work with Cell Culture

I am currently pursuing my masters at Qatar University and I am investigating the incidence of mycoplasma contamination in cell cultures and the techniques frequently used for the detection and elimination of mycoplasmas in research laboratories at Qatar.

I would appreciate your assistance in filling out this survey. Your response will make a difference.

Thank you for your help and your time.

1. Name (Optional):

* 2. Institute:

- Anti-Doping Lab
- Hamad Medical Corporation _ HMC
- Qatar Biomedical Research Institute _ QBRI
- Qatar Cardiovascular Research Center _ QCRC
- Qatar Science and Technology Park _ QSTP
- Qatar University _ QU
- Weill Cornell Medical Collage in Qatar _ WCMC-Q
- Other (please specify)

* 3. What is the focus of your cell culture work?

- Cancer
- Diabetes
- Obesity
- Stem Cell
- Other (please specify)

1

* 4. What type of cells do you work with?

- [ ] (3T3) Mouse Embryonic Fibroblast Cells
- [ ] (ESC) Embryonic Stem Cell
- [ ] (HEK 293) Human Embryonic Kidney
- [ ] (MCF7) Michigan Cancer Foundation-7
- [ ] (MMEC) Mouse Microvascular Endothelial Cells
- [ ] (OVCAR-3) Ovarian Adenocarcinoma
- [ ] (SKOV3) Ovarian Adenocarcinoma
- [ ] Other (please specify)

* 5. Do you experience *Mycoplasma* contamination with your cell culture work?

- ( ) Yes
- ( ) No

If Yes, please move to next question

* 6. Which method do you use to detect *Mycoplasma* contamination?

- [ ] DAPI Staining
- [ ] LookOut® Mycoplasma PCR Detection Kit / Sigma
- [ ] Mycoplasma Detection & Elimination / invivogen
- [ ] MycoAlert™ PLUS Mycoplasma Detection Kit / Lonza
- [ ] Other (please specify)

* 7. How frequently do you test your cell culture for *Mycoplasma* contamination?

- ( ) Weekly
- ( ) Monthly
- ( ) Every other month

* 8. Which method do you use to treat your contaminated cells?

- [ ] BM-Cyclin / Roche
- [ ] LookOut® Mycoplasma Elimination Kit / Sigma
- [ ] Mycoplasma Detection & Elimination / invivogen
- [ ] MycoZap™ Mycoplasma Elimination Reagent / Lonza
- [ ] Plasmocin™ / invivogen
- [ ] Other (please specify)

* 9. When do you use your treated cells?

- ○ 1 passage (sub-culture) after *Mycoplasma* treatment
- ○ 2 passages (sub-culture) after Mycoplasma treatment
- ○ 3 passages (sub-culture) after Mycoplasma treatment
- ○ 4 passages (sub-culture) after Mycoplasma treatment
- ○ Other (please specify)

* 10. Do you use Gene expression analysis as a downstream application of your cell culture work e.g Microarray, RT-PCR or RNA-Seq?

- ○ Yes
- ○ No

3

APPENDIX C: Poster of ARC16 (Annual Research Conference)