INVESTIGATING THE INFLUENCE OF CANNABINOIDS ON MYOBLAST GROWTH AND DIFFERENTIATION

THE EFFECTS OF CANNABIDIOL AND CANNABINOL ON C2C12 MYOBLAST PROLIFERATION AND DIFFERENTIATION

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A thesis submitted to McMaster University

in fulfillment of the thesis requirement

for the degree of Master of Science in Kinesiology

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MASTER OF SCIENCE (2019) Kinesiology	McMaster University Hamilton, Ontario
TITLE:	The effects of cannabidiol and cannabinol on C2C12 myoblast proliferation and differentiation
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NUMBER OF PAGES:	xi, 138

LAY ABSTRACT

Nutrition impacts the regulation of skeletal muscle mass, with many individuals turning to supplements as a means to improve overall health. Cannabidiol – a constituent of the cannabis plant – has been used over the past several decades for its anti-inflammatory, neuroprotective, and anxiolytic properties; however, recent evidence has revealed its potential effectiveness in promoting muscle growth. If true, there is a possibility that it can be used to target the age-related loss of muscle mass, sarcopenia, or even improve athletic performance. Other derivatives, such as cannabinol, have seldom been studied but also demonstrate anti-inflammatory effects. Therefore, this thesis further elucidates the effects of cannabidiol and cannabinol on the myogenic signaling pathway. As a model, we used the murine C2C12 cell line that recapitulates the behaviour of human myoblasts. Interestingly, the data presented herein supports the notion that cannabidiol and cannabinol only promote cell growth and have no effect on myoblast maturation and myotube formation. These findings provide a better understanding of the potential for cannabidiol and cannabinol as a nutritional supplement targeting skeletal muscle.

ABSTRACT

Increasing interest has emerged in the field of nutrition and its role in promoting skeletal muscle growth. Recently, studies using both *in vitro* and *in vivo* models have suggested that cannabidiol – a constituent of *Cannabis Sativa* – can increase the growth and regenerative capacity of skeletal muscle stem cells. Other isolated compounds, such as cannabinol, have demonstrated anti-inflammatory effects *in vivo*. Due to the potential benefits of both compounds, our primary objective was to further elucidate the effects of cannabidiol and cannabinol on murine C2C12 myoblast proliferation and differentiation. We hypothesized that supplementation of cannabidiol and cannabinol would augment gene expression of myogenin, leading to enhanced myotube formation; as well as, induce greater gene expression of Myf5 and MyoD, accompanied by increased cell proliferation. In relation to skeletal muscle growth, myostatin and follistatin can substantially impact the regulation of hypertrophy; with down-regulation of myostatin being a potent stimulus for muscle growth, and follistatin being the antagonist to myostatin, we therefore examined if cannabidiol or cannabinol influenced these two proteins, as a possible rationale for increased myogenesis. In this study, cells were treated with either: (1) cannabidiol, (2) cannabinol, (3) or vehicle control (methanol). Cells were grown for 48 hrs in their respective media, the MTT assay was used to assess proliferation. Muscle differentiation experiments required cells to grow for seven days with media supplemented with the respective compound. The media was changed every 48 hrs. The extent of muscle differentiation was assessed via immunocytochemical and qPCR analysis. In preliminary experiments, cell proliferation was influenced by the duration of which cells were exposed to the compound and concentration of the compound within the media. It was noted that changing growth media and compound every 24 hrs augmented the proliferative response compared to leaving it on for 48 hrs for both cannabidiol and cannabinol ($p<0.05$). Furthermore, supplementing cells with cannabidiol at a 1 or 5 uM concentration resulted in considerable cell growth compared to vehicle control ($p<0.0001$). Cannabinol at 5 uM showed the same effect ($p<0.0001$). We also quantified the mRNA expression of genes involved in the myogenic regulatory pathway in proliferating and differentiating cells. Herein we report that using a 5 uM concentration of cannabidiol or cannabinol did not increase the expression of any of these genes in proliferating or differentiating cells. These findings help further characterize the effects of cannabidiol and cannabinol on the myogenic response.

ACKNOWLEDGEMENTS

To Dr. Gianni Parise – I would first like to thank you for allowing me the opportunity to continue my education in grad school and guiding me throughout my thesis project. After working in cell culture research, it has shaped my interest in the field and has given me a new perspective of potential careers, for which I am grateful for. This study was exciting and extremely interesting to me from the start and I appreciate your trust in me undertaking it. Looking back at the past few years, it has been an incredible learning experience and most rewarding as well.

To my supervisory committee – Dr. Vladimir Ljubicic and Dr. Josh Nederveen, thank you for providing your expertise and guidance throughout this project. I was fortunate enough to have such a respected and friendly group of individuals on my committee that I could learn from and ask advice.

To my mentors – Josh, Jeff, and Sophie thank you for taking the time and patience out of your busy schedules to teach me how to run experiments, use research equipment, analyse data, and apply for scholarships and grad school. The list goes on, but I will always appreciate the effort you put into helping me out, and I hope that I can one day repay the favour.

To Todd, thank you for all the assistance you provided me when equipment seemed to be breaking down or had changed settings. It was incredible to see the effort and focus you put into all of these problems, which helped make this project run a lot more smoothly.

To my research group, I will miss working in the lab with such a smart and talented group of people that made grad school an enjoyable experience with many lasting memories.

To my Mom, Dad, and Sister, thank you for working so hard to put me through school and the support that you have given me. I will always be grateful for having such caring parents that would do their best to help me do well.

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LIST OF ABBREVIATIONS

SC	Satellite cells
CSA	Cross sectional area
MRF	Myogenic regulatory factors
p16	Cyclin-dependent kinase inhibitor
MSTN	Myostatin
GDF8	Growth and differentiation factor 8
TGF-β	Transforming growth factor beta
TA	Tibialis anterior
PI3K	Phosphoinositide 3-kinases
Akt	Protein Kinase B
mTOR	Mammalian target of rapamycin
TSC2	Tuberous Sclerosis Complex 2
S6	Ribosomal Protein S6
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
MPS	Muscle protein synthesis
FSTN	Follistatin
Act RIIB	Activin receptor type IIB
GDF-11	Growth differentiation factor 11
THC	Tetrahydrocannabinol
CBD	Cannabidiol
CBN	Cannabinol
MS	Multiple sclerosis
IFN-y	Interferon Gamma
IL-17	Interleukin 17
PPARy	Peroxisome proliferator-activated receptor gamma
DMD	Duchenne muscular dystrophy
GCPR	G protein coupled receptors
CB1	Cannabinoid receptor 1
GPCR	G protein coupled receptors
cDNA	Complementary deoxyribonucleic acid
ADMETox	Absorption, distribution, metabolism, excretion, transport
Caco-2	Human colon carcinoma cell
TRPV1	transient receptor potential cation channel
RSV	Resveratrol
ERK	Extracellular signal regulated kinase
AMPK	AMP-activated protein kinase

DECLARATION OF ACADEMIC ACHIEVEMENT

For the contents of this thesis, it is prepared in the format outlined by the school of graduate studies, which includes an introduction, detailed methods, results and discussion section. During the project, Sean W. Lau was the principal contributor to completing experiments, data collection, data analysis and interpretation. Dr. Gianni Parise formed the vision of this project, and assisted with interpreting results. Finally, Dr. Sophie Joanisse and Dr. Jeff Baker helped with designing experimental methods and assay validation.

INTRODUCTION

i. The Importance of Skeletal Muscle

In adults, skeletal muscle accounts for ~40% of total body mass and is essential for physical movement, posture, and breathing[1, 2]. Its other roles, though less obvious, include regulating energy and protein metabolism throughout the body[2]. Undoubtedly, muscle has a critical influence on our self-preservation, yet a growing concern has been the age-related decline in muscle mass, otherwise known as sarcopenia[1, 2]. The consequences of sarcopenia can be severe, with many older adults facing a higher risk of physical disability, poor quality of life, and even death. Although the importance of muscle function is understood, the rate at which muscle quality declines with aging can be severely underestimated. It has been shown that by the seventh and eighth-decade of life lean muscle mass can decrease to 25% of total bodyweight[119], with further evidence revealing that in the lower body, such as the vastus lateralis, muscle mass can decrease by 40% between the ages of 20 and 80 years[120]. Even with the benefit of 'healthy aging', individuals still suffer from declining muscle quality due to deterioration of fibre structure, mechanics, and function[16]. When comparing muscle biopsies from healthy active men between 15-83 years of age, it was apparent that there was a greater reduction in overall number of muscle fibres and cross-sectional area (CSA) of older men, type II fibers were particularly affected and were accompanied with more fat and connective tissue[3]. Multiple groups have examined the impact of age on muscle strength. There is a decline in isometric and isokinetic contractions of 20-40% in the knee extensors for those in the seventh and eighth decade of life, while those in the ninth decade experienced an even greater loss of 50% or more[4, 5]. The impact of declining muscle mass in the elderly should not be underestimated. In Canada alone, the economic cost from complications of seniors falling due to a lack of muscle function is two billion dollars annually[6]. Therefore, it is crucial that interventions are identified for ameliorating muscle wasting with advancing age. An area of growing interest is the effectiveness of supplements on improving the myogenic pathway, which can be applied to the progressive onset of age-related muscle loss.

ii. Role of Satellite Cells

Since their discovery in 1961, satellite cells (SC) have been shown to be indispensable for muscle regeneration and remodelling, especially after exercise, trauma, or disease. SC function is dependent on the expression of Pax 7, Myf5, MyoD, Mrf4, and Myogenin, collectively known as the myogenic regulatory factors (MRFs)[22, 23, 24]. Typically, within postnatal muscle, SCs reside in a 'quiescent' state in which they are dormant and express the genetic marker Pax7+[25]. In the event of exercise or traumatic injury, it has been shown that an up-regulation of transcription factors Myf5 and MyoD initiates the progression of SC through the myogenic program. Subsequently, SCs divide and proliferate, either to self-renew or fuse onto existing myofibers or each other to form nascent myotubes. Differentiation requires the expressions of Myogenin and Mrf4[26, 27]. With differentiation, myoblasts fuse to existing myofibers and contribute to the repair and regeneration of muscle. Collectively, the up- and down-regulation of the MRFs are essential for the proper function of SCs. Therefore, myogenic regulators should be investigated when exploring pathways for improving muscle growth and regeneration.

It has been hypothesized that the decreased activity of SCs observed with aging contributes to the onset and progression of sarcopenia[17]. Other identified factors include a reduction in the rate of protein synthesis[7, 8], decreased innervation of muscle fibers[9, 10], loss of mitochondrial function[11, 12, 13], and nuclear apoptosis[14, 15]. The reality is that sarcopenia occurs due to a multitude of factors including a failure of the SC population. SCs present an interesting case in that they are the precursors to muscle fibers and considered to be the primary (or only) contributor of new myonuclei to skeletal muscle fibres[16], making them an ideal target for therapeutic solutions to mitigate muscle loss. Mounting evidence suggests that SC content decreases in skeletal muscle with advancing age. In a study by Verdijk and colleagues (2014), it was reported that with advancing age, not only were type II muscle fibers substantially smaller but aging was also accompanied by a reduction in type II SC content[17]. In addition, it should be noted that from birth to adulthood, there are no considerable changes to the muscle fibre type and SC content[17] suggesting the decline occurs with advancing age. Others have also found a link between SCs and muscle wasting. Brack and colleagues (2005) revealed that a decrease in SC content led to muscle fibre atrophy[18]. This was determined by the initial understanding that muscle fibres are composed of myonuclei, which govern a pre-determined area of cytoplasm, referred to as the myonuclear domain[150]. Based on this theory, they noticed that the nuclei/unit length in aging muscles decreases in larger fibers. In parallel, SC content also declines with age, causing a decrease in fibre size to compensate for the standard myonuclear domain range[18]. In relation to SC content and strength, Verdijk and colleagues (2010) examined muscle biopsies, as well as leg strength from older men (<65 yrs), and demonstrated a strong correlation between muscle mass and strength with overall SC content and fibre CSA[19].

Besides overall SC number, deficiencies in SC function are also believed to strongly impact the onset of sarcopenia. It is well established that older adults have an impaired recovery of muscle mass and strength after an acute bout of immobilisation compared to young adults[121, 140]. However, the underlying cellular mechanisms that lead to impaired recovery with aging remain unknown. Suetta and colleagues (2013) were able to further elucidate the dysfunction by analysing the expression of MRFs in healthy young and old males following leg immobilization for two weeks and retraining for four weeks. It was reported that even with re-training, older males had no detectable gains in myofiber area (MFA) or SCs, whereas younger males increased their MFA and had more SC per type II fibre. The impaired muscular recovery in seniors noted by Suettta and colleagues may be attributed to an impaired response in SC proliferation[20]. A study done by Sousa-Victor and colleagues (2014) compared SC function of young, old and geriatric mice. It was apparent that SCs from geriatric mice lost the ability to transition out of quiescence and entered an irreversible senescent state, caused by the de-repression of p16INK4a, ultimately reducing regenerative and self-renewal capacities. Remarkably, even with injury, these cells were unable to activate and expand, while further accelerating towards full senescence[21]. Overall, these studies provide evidence that either

SC content or function can have an impact on the age-related decline in muscle mass and strength.

iii. Myostatin and Muscle Growth

Myostatin (MSTN) – also known as growth/differentiation factor 8 – is a protein found within the transforming growth factor beta (TGF-β) superfamily that has the ability to highly regulate muscle mass in animals and humans. For this reason, it has garnered considerable interest as a target for pharmacological interventions. A wealth of literature has shown that the decreased expression of MSTN can promote significant muscle growth, while overexpression can lead to muscle atrophy[28, 29]. To determine the regulatory effects of MSTN on cellular pathways, the application of cell culture techniques have been commonly used in studies as physiologically accurate and reliable models. In 2000, Thomas and colleagues presented early evidence that MSTN functions by controlling the proliferation of muscle precursor cells[141]. In their findings, incubation of MSTN led to decreased proliferation of murine myoblast cells, which stemmed from cell cycle arrest in the G1 phase[141]. Further analysis indicated that MSTN specifically up-regulated p21 – a cyclin-dependent kinase inhibitor – and decreased the level and activity of Cdk2 protein in myoblasts[141]. A few years later, the same cell model was used to further examine MSTNs influence on the regulation of MRFs[142]. It was discovered that increasing concentrations of recombinant MSTN repressed protein levels of MyoD, Myf5, myogenin, and p21, which was mediated by the protein Smad-3, leading to the inhibition of myogenic differentiation[142]. On the other hand, the inhibition of MSTN in cultured SCs results in an in increase myotube formation, indicating its vast influence on myogenesis[143]. Indeed, MSTN has a powerful impact on regulating skeletal muscle mass, which is apparent in gene knockout models of numerous animals. Specifically, Welle and colleagues (2007) demonstrated that reducing MSTN mRNA expression by less than 1% in mice resulted in ~25% increased skeletal muscle mass within 3 months[30]. Remarkably, this genetic mutation can also occur naturally and has produced hyper-muscular phenotypes in mice, sheep, dogs, humans, and some cattle breed[32, 33, 34, 35, 36].

It has been suggested that endogenous levels of MSTN may influence the prevalence of sarcopenia[37, 38], however, the method of measuring MSTN concentration is complex, and there are various studies showing mixed results on the topic[39, 40, 41]. Research in support of this theory demonstrates that older adults exhibiting a decline in muscle mass have an up-regulation of MSTN protein in blood when compared to younger subjects[42, 43, 44]. Indeed, further examination was merited on the pathways linked to this negative-regulator of muscle mass. McKay and colleagues (2012) reported a link between MSTN and SCs after a bout of resistance exercise. Specifically, at baseline the number of SCs colocalized with MSTN was not different between old and young men, however, 24 hrs after a single bout of unilateral loading the proportion of type II fibre-associated SC colocalized with MSTN was 67% higher in older men. This was accompanied by a severely blunted progression of SCs through the myogenic program, suggesting that the increased colocalization of MSTN in SCs induced impairment in myogenic capacity of aged muscle[44]. It has also been suggested that the inhibition of MSTN may have protective qualities in aging animals experiencing muscle wasting. In 2006, Siriett and colleagues were able to show that old *MSTN*-null mice had reduced age-related muscle loss than their wild-type counterparts. These mice expressed little to no fibre type shifting and minimal atrophy, whereas old wild-type mice showed a greater transition to oxidative fibre types, as well as more atrophy[45]. Taken together, the above findings illustrate MSTN as a critical regulator of the myogenic process and can be a beneficial target for increasing muscle mass.

iv. Follistatin and Muscle Growth

Follistatin (FSTN) is a protein that has emerged as an active antagonist to the up-regulation of MSTN. Its expression can be found in nearly all tissues within the body where it binds and neutralizes numerous members of the TGF-β superfamily[47, 48]. Likewise, FSTN has a strong affinity for MSTN and is capable of binding and preventing downstream MSTN signalling[121]. The functional significance of this protein was determined using transgenic mice that expressed high levels of MSTN, compared to ones injected with FSTN cDNA[49]. After analysing protein regulation, it was concluded that FSTN binds onto the C-terminal dimer of MSTN and inhibits its ability to bind to receptors, resulting in a dramatic increase of muscle mass even compared to MSTN-null mice[49]. Furthermore, when FSTN over-expressers were crossed with MSTN knock out animals there was an additive effect on muscle mass with a quadrupling of muscle mass. Together, the results demonstrate that FSTN inhibits MSTN, but also influences other pathways regulating muscle mass. In summary, FSTN possesses potent myostatin inhibiting characteristics that can have a powerful influence on muscle regeneration and growth.

v. The variability in response to supplements

Nutritional supplements are an efficient and easy dietary additive for individuals looking to improve overall health. Supplements are sold in seemingly endless forms and compositions with the added factor that they can be specific for age, gender, and athletes[58]. For many, it can be challenging to determine the extent of which these products are beneficial, primarily because of the potentially small effect supplements may give off [62]. There are supplements in the market, such as whey protein, that have shown to be effective in improving the rate of growth of skeletal muscle following feeding and exercise[128, 129, 130, 131]. Yet, other compounds commercialized for the same purpose, like BCAAs or testosterone, show insufficient data or have no myogenic effect at all[125, 126, 127]. It is understandable that such discrepancy exists in over the counter supplements. Within Canada, the requirements for monitoring and evaluating natural health products are loosely regulated and considered by the natural health regulations as low-risk products[63], without a priority on efficacy. In comparison to pharmaceutical products, these guidelines require minimal evidence to support their statements and can often have little to no experimental research to support their claims[63]. There is a significant number of individuals taking supplements, with estimates that ~45% of the population in Canada use at least one supplement a year[57], which has been a growing trend during the past few decades[124]. This progressive rise in the use of supplements can be attributed to a number of factors including an aging population of 'baby boomers' concerned over their wellness and health, as well as a growing cohort of older adults experiencing chronic illness[58]. Be it as it may, there are a considerable number of individuals consuming supplements to better their health, but may not be receiving any benefits at all from these supplements.

vi. Overview of Cannabinoids

In the past decade, cannabinoid chemistry and pharmacology have become increasingly prevalent in research and have been the focus of thousands of publications. To this day, researchers determined there are over 110 different phytocannabinoids isolated from the plant *Cannabis Sativa*[65]; most of them, are similar in chemical structure but exhibit various physiological responses when consumed[132]. Out of its many constituents, Δ9-tetrahydrocannabinol (THC) is the most recognized due to its popularity in eliciting euphoric effects, leading many to believe it is the only factor responsible for the effects of cannabis. However, mounting evidence has proven other isolated components of the plant can provide therapeutic effects, such as anti-inflammatory [66, 67], neuroprotective[68], and anxiolytic properties[69, 70, 71]. Indeed, cannabidiol (CBD) – a non-psychoactive compound – has gained considerable interest for its ability to regulate muscle regeneration and growth. Specifically, it was shown by Giacoppo and colleagues (2016) that CBD has a positive effect on the Akt/mTOR pathway in mice with experimental multiple sclerosis (MS). In their study, CBD (10mg/kg) was administered for 14 days after the symptoms of MS started to appear[72]. Evidently, mice that had symptoms of MS and were treated with CBD showed significant up-regulation of PI3K/Akt/mTOR proteins compared to their wild-type counterparts[72]. Further evidence also revealed that the administration of the compound caused a greater potential for muscular recovery by reducing pro-inflammatory cytokines, such as IFN-y, IL-17, and increasing PPARy[72]. With the current understanding that the mTOR complex plays a pivotal role in promoting muscle protein synthesis (MPS), it can be hypothesized that through its increased activation there may also be an enhanced myogenic response. In addition, other studies have found a significant effect of CBD on muscle regulation using murine C2C12 myoblasts, primary SCs, as well as myoblasts from healthy and duchenne muscular dystrophic (DMD) patients[73]. Their comprehensive investigation revealed CBD as a promoting factor for differentiation in C2C12s and myoblasts isolated from healthy and DMD donors[73]. It was discovered that the underlying mechanisms centralized around an increase in calcium uptake through the transient receptor potential channels, providing further evidence of the potential structures that CBD targets[73]. In dystrophic mice, the administration of CBD (60mg/kg) helped prevent the loss of locomotor activity, reduced inflammation, and restored autophagy, commonly associated with the disorder[73]. In contrast to the wealth of knowledge surrounding CBD, cannabinol (CBN) – a mildly psychoactive cannabinoid found in trace amounts[147] – has been seldom studied. Although little is known about CBN, one study demonstrated its physiologically therapeutic capabilities that could potentially enhance regeneration of muscle. Using the carrageenan induced paw edema model – a popular test used for screening anti-inflammatory activity – it was reported that CBN was effectively able to reduce collagen-induced arthritis in rat models[148]. While more research is still needed on the functional relevance of CBN, it would be interesting to examine its influence on myogenic regulation.

It was revealed recently that the biochemical reactions involved around cannabinoids are primarily through receptors found on cell membranes[74, 75], which are known as *G Protein Coupled Receptors* (GCPRs). These are classified as either CB_1 or CB_2 and can be found in various concentrations throughout the body[75]. CB_1 has proven to be the most widely expressed receptor protein and is present in several regions of the brain, such as the cerebellum, hippocampus, basal ganglia, amygdala, hypothalamus, and brainstem[144]. In addition to the brain, CB_1 is also highly expressed in the peripheral nervous system and in most mammalian tissues and organs (i.e. heart, liver, adipose tissue, lungs, skeletal muscle) [144]. The activation of CB_1, either by natural or synthetic ligands, has been reported to influence a host of homeostatic functions, some of which include: regulating the psychoactive potential from exogenous cannabinoids[76, 130], modulating the mobility of the GI tract[144], or increasing permeability of the intestinal epithelium[144]. In contrast to the abundant expression of CB_1, CB_2 receptors are reported to have a lower quantity by up to 100-fold[145], due to its rarity, on-going research is continuously finding new locations of the receptor. Currently, literature has proposed that CB_2 is predominantly expressed in cells associated with the immune system, and in other peripheral tissues, including the cardiovascular system, GI tract, liver, and adipose tissue. Because no CB_2 receptors are found in the CNS, it is referred to as "the peripheral cannabinoid receptor" [144]. Their reaction to binding endocannabinoids under various pathological conditions or disease states appears to give off immunosuppressive properties such as anti-inflammatory signals[77]. Interestingly, exogenous CBD has little affinity for either of the cannabinoid receptors but acts through various receptor-independent pathways such as TRPV1 and serotonin receptors[73, 133, 134]. On the other hand, CBN has been noted to act as a partial agonist at the CB_1 receptors with even higher affinity to CB_2 receptors[149]. Naturally, scientists have found endogenous cannabinoids that activate both CB_1 and CB_2 receptors via paracrine signalling and serve as intercellular 'lipid messengers'[78]. A unique feature of these endogenous cannabinoids is their ability to exist as precursors on cell membranes, which can be cleaved by specific enzymes when needed[79, 80, 81]. Compared to other neuromodulators, this form of synthesis allows for signalling to occur on demand rather than made and stored for later use[79, 80, 81]. Their production involves a variety of physiological functions, including appetite, pain sensation, mood, and memory[82], making this system valuable for drug and therapeutic research. Overall, cannabinoid receptors can influence cells in a variety of ways, and CBD has demonstrated its unique potential to limit muscle degeneration that may truly benefit human health.

vii. The Significance of Cell Culture Research

Since the advancement of cell culture techniques in the 1950s, it has become increasingly prevalent in biological experiments, especially as it is applied to human health. Through its application, there have been ground-breaking discoveries for viral vaccines, monoclonal antibodies, and recombinant therapeutic proteins[83]. Indeed, cell culture is a physiologically reliable model that can be used for investigating the biophysical and biomolecular mechanisms in cell and drug therapy. Its significance has been shown in determining the effectiveness of drug absorption, distribution, metabolism, excretion, and toxicity (ADMETox)[135]. Countless studies have used various cell types grown in a 2D model to investigate the different aspects of ADMETox. For instance, the human colon carcinoma cell (Caco-2) has been commonly used to predict intestinal drug permeability and absorption in humans[84]. Cultured Caco-2 cells express distinct characteristics of intestinal epithelium, such as brush border microvilli, dome formation, and tight bonds amongst each other[84, 85]. Furthermore, these cells produce proteins capable of transporting chemical substances, making them also well suited for testing drug transport[86]. In relation, Alhamoruni and colleagues (2010) were able to determine the intestinal permeability of CBD using the caco-2 cell model. Permeability was measured using transepithelial electrical resistance, with potential target sites such as the CB_1 and CB_2 receptor, TRPV1, PPARγ, PPARα, and other proposed cannabinoid receptors. By using ethylenediaminetetraacetic acid to increase abnormal levels of permeability, CBD was able to provide relief to the high rate of permeability, suggesting that it may have therapeutic potential for highly permeable intestinal epithelium[87]. Other cells, such as the Madin-Darby Canine Kidney, have an interesting capacity of releasing P-glycoprotein, a plasma membrane protein that acts as a drug transport mechanism by exporting drugs out of cells, which decreases the intracellular concentration and provides faster results in drug transport assays[88]. In similar cases, many researchers have found that the immortalized cell line HepG2 – derived from primary hepatocytes – can accurately test drug metabolism and toxicology before beginning clinical trials[89]. Its application has been crucial for detecting drug-induced liver injuries while also being termed the gold standard for xenobiotic metabolism and cytotoxicity studies[136]. In a revision of toxicity testing done by the U.S National Research Council, it has been calculated that out of the 51 drugs taken out of distribution, 29 were withdrawn due to hepatotoxicity and cardiotoxicity in HepG2 cell models[137], demonstrating its applicability for identifying risk assessment. Overall, these cellular assays are examples of the significant impact cell culture may have on drug screening and development, as well as its vast experimental capabilities.

Most mammalian cell culture uses either primary or established cell lines grown in a suitable culture vessel with media. The origin of primary cells are directly isolated from either tissue or cell suspension and have limited growth before reaching senescence[83]. This 'biological clock' is attributed to chromosomal length, which was noted by Cooke & Smith in 1986; their study reports unequal caps, also known as telomeres, at the end of chromosomes in human germline cells compared to somatic cells. These were later determined to be repeats of the nucleotide sequences that became shortened after each stage of proliferation[90]. In light of this, scientists were able to find a solution by transforming primary cells into a continuous cell line; whereby, cells would proliferate indefinitely. Currently, there are various techniques that can be used to develop an immortalized cell line, including mutagens, viruses, or oncogenes[91]. As a result, numerous established cell lines exist today using different cell types (i.e. fibroblasts, myoblasts, epithelial cells), with the additional benefit that they can be grown in two-dimensional (2D) or three-dimensional (3D) models. Cells in 2D, are plated as a monolayer, primarily using a petri dish or culture flask[113]. Advancements in biotechnology have produced 3D models that can grow cells in multiple ways, including:

forced floating[92], hanging drop[93], agitation-based approach[94], matrices[95], scaffolds[96], and microfluidic platforms[95]. Comparing the two, there is a debate on which provides greater physiological relevance, albeit more literature has supported 3D models[97]. Karlson and colleagues (2012) were able to further examine the differences, by using colon cancer cells (HCT-116) either grown as 3D spheroids or in monolayer. Their application of six standard anti-cancer drugs showed that cells in 2D had an extremely high response, which was unfeasible if used in *vivo.* Meanwhile, cells in 3D spheroids had a blunted response from the treatment, which was comparable to its administration in humans[98]. There are several explanations why this model may better predict results in *vivo.* It has been shown that a limited diffusion of compounds through the spheroid can better imitate features of solid tissue, while also fluctuating the availability of oxygen, nutrients, metabolites, and signaling molecules[98]. Additionally, the 3D model can provide cell-cell and cell-environment interactions responsible for cellular decision-making[98]. These characteristics of a 3D model give results more validity, although, others have also proposed that 2D cell culture can be more relevant than its counterpart. It was Ikeda and colleagues (2017) that studied the contractile force generation of tissue-engineered skeletal muscle using C2C12 myotube differentiation in 2D and 3D models. They found the levels of contractile force within cells grown in 3D were not correlated with levels from skeletal muscle constructs[100]. On the other hand, sarcomere function and contractile activity of those in 2D cell culture showed significant resemblance[100]. Therefore, careful consideration should be made into the specific cell model used during experimental application.

Assays have been developed to determine the effectiveness of nutritional compounds in both 2D and 3D models. These methods are evident in a study by Monesano and colleagues (2013), with the evaluation of resveratrol (RSV) on proliferation and differentiation of murine myoblasts. Using immunoblotting analysis, they determined that RSV promotes myogenesis and hypertrophy by influencing protein synthesis and MRFs protein expression. Specifically, RSV was shown to stimulate the IGF-1 signalling pathway, by increasing AKT and ERK 1/2 protein activation, as well as AMPK protein abundance, and decreased the gene expression of myogenic markers Myf-5 and MyoD[101]. RSV a natural polyphenol found in grapes and other fruit is believed to provide immune regulation, DNA repair, cancer chemoprevention, cardio, and neuro-protection[101]; however, it has not been known to increase myogenesis until this investigation. Therefore, cell culture has its role in laying the foundation for *in vivo* experiments and is one of the major tools used in cellular and molecular biology. These examples provide a small glimpse at the capability of testing nutritional compounds, which can further be elucidated in animal and human trials.

viii. Physiological Relevance of Murine C2C12 Myoblasts

A common model used for exploring myogenesis and the expression of target proteins is the immortalized C2C12 cell line. As a subclone of mouse myoblasts, they are capable of rapid proliferation and maturation into functional skeletal muscle, making them ideal for investigating the effects of CBD on myogenesis[113]. Yaffe and Saxel (1977) reported that within four days of differentiation, multinucleated myotube networks formed, and a few days later, sarcomeres and z-lines could be seen[102]. These physiological structures formed by C2C12s help provide an accurate representation of skeletal muscle found in humans. Other studies have noted that C2C12s express myofilament proteins important for muscle contractions, these include: slow-twitch skeletal muscle, embryonic and perinatal myosin heavy chain isozymes[103], slow and fast-twitch troponin I isoforms [104], cardiac muscle troponin C isoforms[105, 106, 107], and striated muscle tropomyosin[110]. Altogether, these findings imply that C2C12s are reliable for assessing the biomolecular and biophysical effects of CBD and CBN. Modifications can also be made to C2C12s for experimental purposes. To identify hypertrophy-inducing agents for the treatment of sarcopenia, Cross-Doersen & Isfort (2003) refined the cell model by fusing β-myosin heavy chain gene regions to a luciferase reporter gene. This resulted in a cell capable of expressing hypertrophic agents seen in skeletal muscle, including insulin, IGF-1, and testosterone, for both proliferative and differentiated states[111]. These abilities to modify the cell line help to better understand the hypertrophy inducing process, while also demonstrating the various applications of C2C12s.

There are advantages and disadvantages when using an established cell line instead of primary cells. Careful consideration should be made when selecting cell types (C2C12 or primary myotubes) for the experiment in question. Myotubes derived from primary cells show a higher assembly of sarcomeres and contractile activity[112]. In addition, they express higher levels of structural components within muscle tissue such as myosin heavy chain, cytochrome C oxidase IV, and myoglobin[112], suggesting its enhanced genetic integrity, and ability to exhibit normal physiological structure and function. However, primary cells can produce greater variability in experiments due to differences in donors, as well as the added difficulty of harvesting cells[113]. They are also hard to maintain with the need to optimize specific culture conditions and slower cell growth, making large-scale experiments unlikely[113]. Moreover, as mentioned earlier, primary cells have a limited lifespan and will reach senescence after a certain number of cell divisions. On the other hand, C2C12s are capable of proliferating indefinitely with a high rate of consistency and have well-established conditions for growth. Other advantages include their fast proliferation rate and relatively low cost, allowing for higher data throughput[113].

ix. Study Objective and Hypotheses

The purpose of this investigation was to evaluate the effects of CBD and CBN on proliferation and differentiation using the murine myoblast C2C12 immortalized cell line. In doing so, we aimed to determine whether CBD or CBN could impact skeletal muscle growth in hopes of translating these results to *in vivo* models for improving athletic performance or prevent age-related muscular diseases such as sarcopenia. Based upon previous literature[72, 73, 146], we hypothesized that CBD and CBN can augment the expression of Myogenin, leading to significant myotube formation; as well as, increase gene expression of Myf5 and MyoD, accompanied by significant cell proliferation. In relation to skeletal muscle growth, MSTN and FSTN have a substantial impact on the regulation of hypertrophy [30, 33, 34, 49, 50], we therefore sought to determine if CBD or CBN can influence these two proteins, as a possible rationale for increased myogenesis.

METHODS

Cell Culture and Reagents

Murine C2C12 myoblasts (cat. n. CRL-1772; ATCC: The Global Bioresource Centre) were cultured in a growth medium (GM) composed of Dulbeco's Modified Eagle Medium (DMEM) High Glucose 1x (cat. n. 11995093; Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) Gibco (cat. n. 12383020; Invitrogen), and 1% penicillin/ streptomycin (cat. n. 15140122; Invitrogen). Proliferating C2C12s were induced into differentiation by exposure to differentiation medium (DM), DMEM containing 2% Horse Serum (cat. n. 16050122; Invitrogen), and 1% penicillin/streptomycin. Depending on the experiment, CBN solution (cat. n. C-045-1ML; Sigma-Aldrich), CBD solution (cat. n. C-045-1ML; Sigma-Aldrich), or Methanol (cat. N. CABDH1135-4LP; VWR) were supplemented into the media. With all media listed above being filtered using Corning Disposable Vacuum Filter/Storage (cat. n. 28199-784; VWR).

General cell culture methods

C2C12 myoblasts were thawed and placed in a falcon tube containing 10ml of GM, then centrifuged at 1500 rpm for 5 min at 20°C[158]. Next, supernatant was aspirated and the cells were resuspended in GM and pipetted onto a cell culture plate[158]. All cells grew in an incubator at 37°C and 5% CO2[158]. In the event that cells needed to be passaged, trypsin 10x solution (cat. n. 15090046; Invitrogen) diluted to 1X with PBS was pre-warmed to 37°C, then GM was removed from the plate and cells washed twice in PBS and 1X trypsin was added (0.5 ml/10cm^2)[159]. Afterwards, the cells were incubated at

37°C for 5 minutes and GM was re-added to deactivate trypsin[159]. The cell suspension was transferred to a tube and centrifuged at 1500 rpm for 5 minutes[159]. After removal of the supernatant, the cell pellet was resuspended in GM and plated onto a dish at the desired cell density[159]. Cell counting involved using the Invitrogen Countess I automated cell counter machine, as well as cell counting slides (cat. n. 10027-446; VWR) and Trypan blue dye (cat. n. 15250061. Fisher Scientific); to perform this test, 10ul of cells combined with 10ul of Trypan blue were mixed together then placed into the well of the slide and placed into the counter[160]. For differentiation experiments, cells needed to grow until 80% confluent then differentiation media (DM) was added and/or changed every 48 hours for 7 days[158].

Cell Proliferation Assay

To assess myoblast proliferation, cells for the MTT assay were plated on a 96 well plate at a density of 1500 cells/well. Quantification of cell growth was determined using an immunofluorescence proliferation assay with a tetrazolium dye known as MTT 3-(4,5-dimethlythiazol-2-yl)-2,5-diphenyltetrazolium bromide[157]. Usage of Yellow MTT theoretically assesses the increase in the number of cells via mitochondrial quantity– i.e. a greater number of cells can convert more yellow MTT to purple formazan through the mitochondria – which has been analysed using the MTT stock solution, 1mL of sterile phosphate buffered saline (PBS) was added to a 5mg vial of MTT, and then dissolved by vortexing[157]. The timeline for the proliferation assay is as follows: cells were added onto a 96 well plate. 24 hours later the media was changed to GM + compound (CBD, CBN or methanol). On the third day 20ul of 5mg/ml MTT was pipetted into each well – one row of wells having MTT but no cells (control) [157]. The plate was incubated for 3 hours at 37°C in a culture hood, media was removed and 150ul of MTT solvent was added[157]. The culture plate was covered with tin foil and placed on an orbital shaker for 15min[157]. Finally; absorbance was measured on the Synergy™ Mx (serial n. 267174; Biotek) at 590nm with a reference filter of 620nm. To determine if incubation time using GM + compound influenced cell growth, a time course experiment was run whereby three separate groups were incubated for 48 hrs as follows: (1) GM + compound for 8 hrs, then replaced with regular GM (2) GM + compound changed every 24 hrs (3) GM + compound remained on for 48 hrs (*see diagram 2)*. For qPCR experiments, cells were added onto 6 well plates at 50 000 cells/well with a replicate for each sample. When ~40% confluent, media was changed to GM + compound (CBD, CBN or methanol) for each well at a 5 uM concentration *(see diagram 1).*

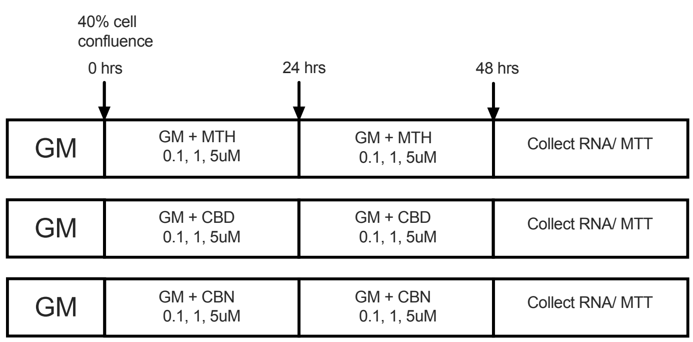


Diagram 1: diagram of the concentration experiments for proliferating cells

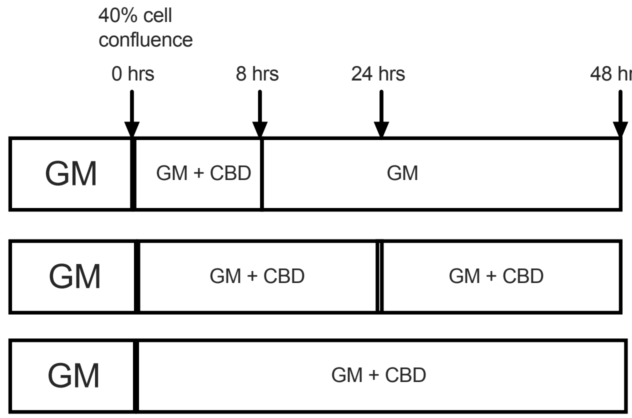


Diagram 2: diagram of the time course experiments for proliferating cells

Cell differentiation assay

For differentiation, cells were plated onto 6 well plates at 100 000 cells/well with a replicate for each sample. C2C12 differentiation required cells to grow at a minimum confluence of 80% on the culture dish before switching to DM. 24 hours later after changing GM to DM (Day 1), DM + compound (CBD, CBN or methanol) was added and changed every 48 hours for a total of 7 days. On the final day immunocytochemical protocols or RNA isolation was completed *(see diagram 3)*.

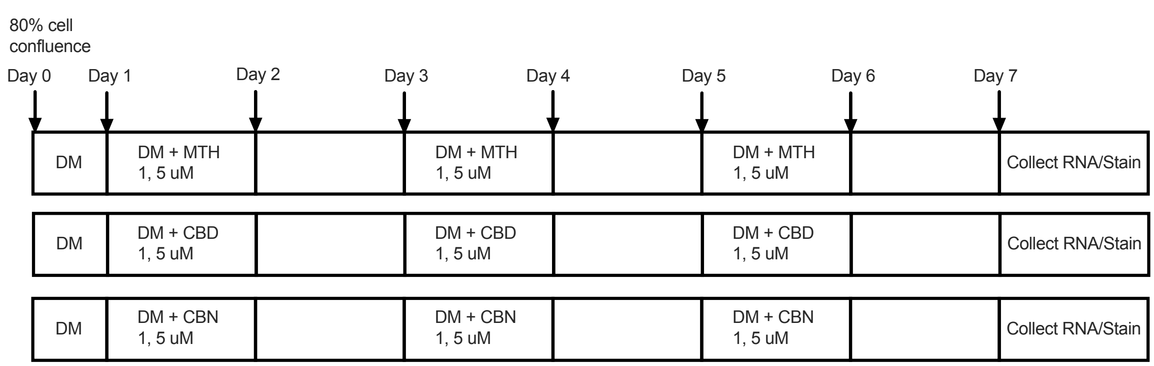


Diagram 3: diagram of differentiation timeline using different concentrations and compounds

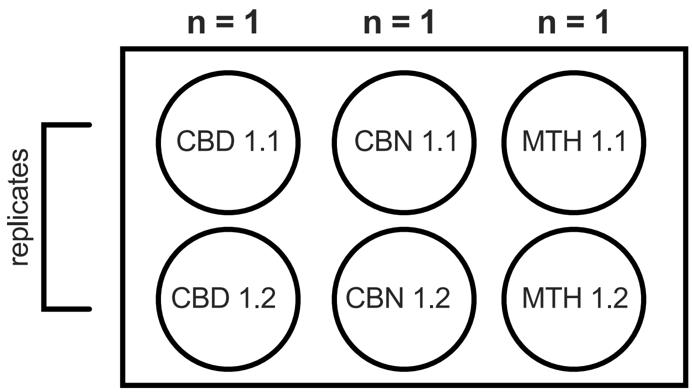


Diagram 4: diagram of a 6 well plate used for both proliferation and differentiation qPCR/immunocytochemistry experiments

Immunofluorescence assay and analysis

With immunofluorescent analysis, MHCI (cat. n. BA-F8-s; University of Iowa) was used to detect myotubes and DAPI (cat. n. D9542-10MG; Sigma Aldrich) to detect nuclei. The staining protocol was as follows: myotubes were fixed on a culture plate with 4% PFA for 30 minutes, then washed with PBS for 3 x 5 minutes. The PBS was removed and 0.1% Triton X in 1% BSA was added for 15 minutes. After, PBS was applied for 5 minutes. PBS was aspirated and cells were blocked in PBS with 5% GS for 1 hour. Block was removed, 1°Ab MHCI (DSHB; clone 5.8, mouse) was added undiluted (neat) and incubated overnight at 4°C. Next day, 2°Ab 488 goat anti-mouse (cat. n. A-21141, Thermo Fisher Scientific) diluted to 1:250 in PBS was placed on the dish and incubated for 2 hours at room temperature (RT). The plate was then washed with PBS for 3 x 5 minutes, after which, DAPI was added for 10min and finally washed again with PBS for 2 x 5 minutes.

Images were captured using the NIKON Eclipse Ti microscope at 10x magnification on the FITC and DAPI channel, taking 4 randomized images per well. Analysis of each image consisted of three different measurements using the NIKON elements application. First, myotube diameter required measuring 5 sections along the width of each myotube. Second, myotube surface area used binary thresholding of overall MHCI (FITC) coverage in the entire image. Finally, myonuclear index used the binary thresholding setting for nuclei, which was then calculated by:

$$\frac{nuclei\ in\ myotubes}{total\ number\ of\ myonuclei}\ x\ 100 = myonuclear\ index\ \%$$

RNA extraction and Quantitative Polymerase Chain Reaction

RNA isolation began with aspirating media and washing with PBS. Trizol reagent (cat. n. 15596018; Invitrogen) was added at a ratio of 0.3-0.4ml per 1 x 10^5-10^7 cells then scraped and pipetted into tubes[154]. Samples were either frozen at -80°C or continued onto the next step[154]. Chloroform was added at 200 uL/mL of Trizol reagent. Tubes were shaken for 15 seconds and incubated at RT for 5 minutes[154]. They were then placed in a centrifuge and spun at 12 000g in 4◦C for 10 minutes[154]. The upper aqueous layer was pipetted to a new tube and an equal volume of 100% ethanol was added[154]. Samples were transferred into RNA spin columns placed on top of 2 mL tubes, both acquired from the E.Z.N.A.® Total RNA Kit I (cat. n. R6834-02; VWR)[155]. Tubes were centrifuged at 10 000g for 60 seconds in RT[155]. Flow through was removed and 250uL of wash buffer I was added on the spin column membrane; centrifuged at 10 000g for 60 seconds in RT[155].

Repeated previous step again, but with 500 uL wash buffer I[155]. Flow through was discarded and 500uL wash buffer II was added onto spin column membrane[155]. Tubes were centrifuged at 10 000g for 60 seconds in RT[155]. Repeated previous step again. Discarded flow through then centrifuged spin column at max speed to completely dry the matrix[155]. The RNA spin column was transferred to a clean 1.5ml collection tube and pipetted 40uL of DEPC-treated water directly onto centre of tubes, incubating for 1min in RT[155]. Centrifuged for 2 minutes at 10 000g. Once complete, RNA content was quantified using the Nano Drop Spectrophotometer[155].

The high-capacity cDNA reverse transcription kit (cat. n. 4368814; Fisher Scientific) was used to perform RT-qPCR. Kit components *(shown in table 1)* were thawed on ice then master mix was made [156]. 10ul of master mix was pipetted into each individual tube mixed with 10ul of RNA sample[156]. Tubes were placed into the thermal cycler and run with parameters described in table 2[156].

Table 1. volume of reagents for cDNA reverse transcription

Component	**Volume for 1 reaction**
10X RT Buffer	2.0 uL
25X dNTP mix (100mM)	0.8 uL
10X RT Random Primers	2.0 uL
MultiScribe Reverse Transcriptase	1.0 uL
Nuclease free H_20	4.2 uL

Total per reaction	10.0 uL

Table 2. Settings for cDNA reverse transcription thermal cycler

Settings	**Step 1**	**Step 2**	**Step 3**	**Step 4**
Temperature	25 ºC	37 ºC	85 ºC	4 ºC
Time	10 minutes	120 minutes	5 minutes	∞

SYBR green assay (cat. n. 330500; Qiagen) was used for quantifying gene expression. Myf5, MyoD, Myogenin, MSTN and FSTN were the genes of interest, while RPS11 was used as a housekeeping gene, all primer sequences are included in table 3. All experiments were run on 384 and 96 well plates with sample duplicates and quantified using the QuantStudio5 real-time (serial n. 272530039; Thermofisher) and Mastercycler Realplex 4 (serial n. A242225G; Eppendorf) PCR machine.

The Livak method was used to analyze the expression of genes. The following of which was calculated by:

$$CT\ target\ gene - CT\ reference\ gene = normalized\ CT$$

$$\Delta CT\ test\ sample - \Delta CT\ calibrator\ sample = \Delta\Delta CT$$

$$Fold\ change = 2^{-(\Delta\Delta CT)}$$

Table 3. Primer sequences for qPCR using SYBR green assay

Gene Products	Forward Primer	Reverse Primer
RPS11	CGTGACGAACATGAAGATGC	GCACATTGAATCGCACAGTC
Myf5	TGAAGGATGGACATGACGGAG	TTGTGTGCTCCGAAGGCTGCTA
MyoD	TACCAAGGTGGAGATCCTG	CATCATGCCATCAGAGCAGT
Myogenin	CTACAGGCCTTGCTCAGCTC	AGATTGTGGGCGTCTGTAGG
Follistatin	AAAACCTACCGCAACGAATG	GGTCTGATCCACCACACAAG
Myostatin	AATCCCGGTGCTGCCGCTAC	GTCGGAGTGCAGCAAGGGCC

Data Analysis

All data are presented as mean ± standard error means. Data was analysed on GraphPad Prism program version 8.0. A two-way ANOVA was used to assess the effect of time course and compound for the MTT assay, all other experiments were analysed using a one-way ANOVA. Both analyses were followed by Tukey *post hoc* analysis to detect statistical significance between three or more independent groups. Statistically significant differences were accepted when P was < 0.05.

RESULTS

Proliferation rate increases after changing media and compound every 24 hours.

It was determined that replenishing GM + CBD (5uM) every 24 hrs resulted in greater proliferation rates compared to leaving GM + CBD on for 48hrs ($p<0.05$) assessed via an MTT assay ($n=8$) **(Figure 1A)**.

Results indicate that replenishing GM + CBN (5uM) every 24 hours produced greater proliferation of myoblasts compared to leaving GM + CBN on for 48 hrs ($p<0.05$). CBN at 5uM also increased proliferation rates significantly when added for only 8 hours, versus 48 hrs ($p<0.05$) ($n=8$) **(Figure 1B)**.

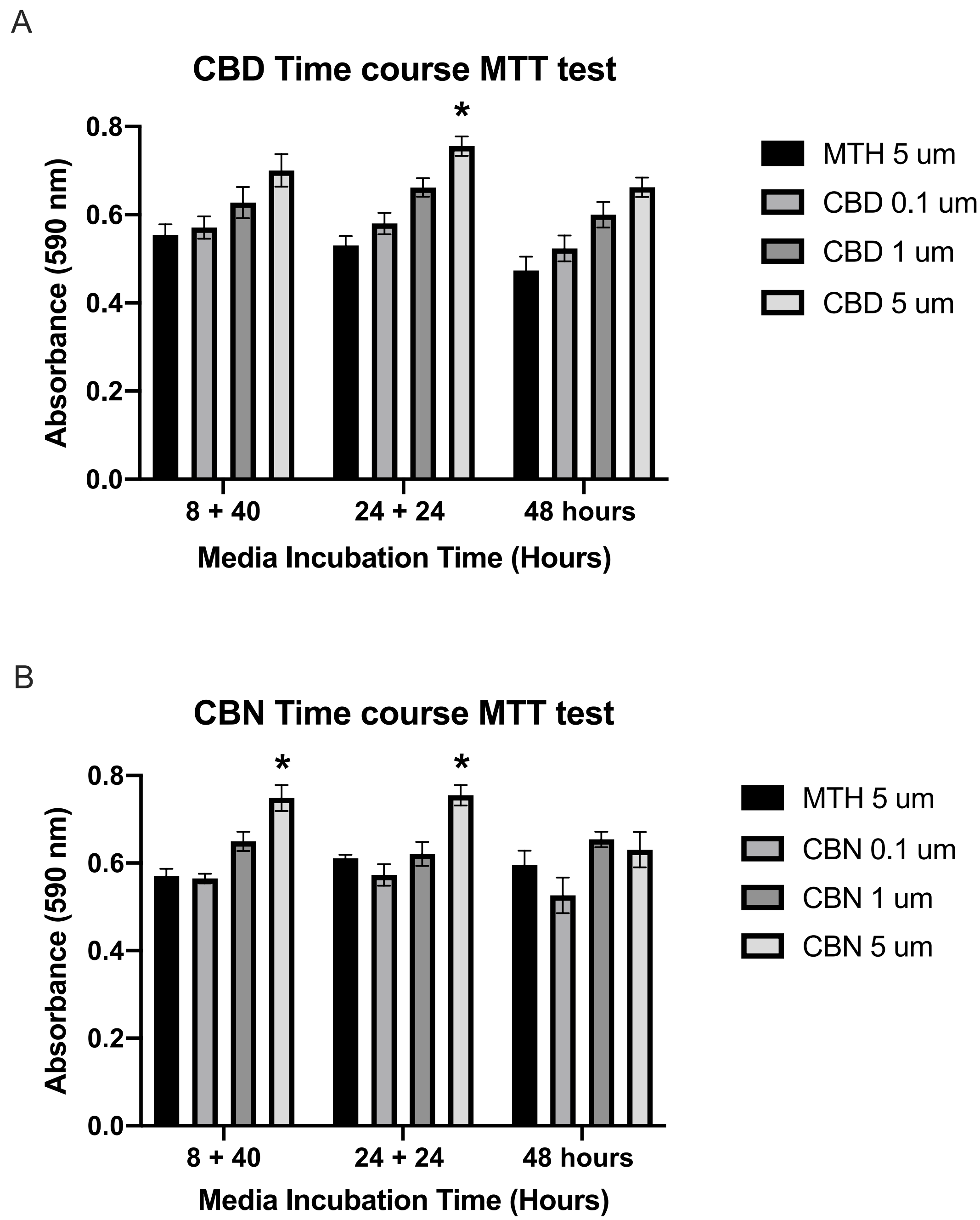


Figure 1. Proliferation of C2C12 myoblasts supplemented with CBD or CBN at different durations and concentrations. The 8+40 group had GM + compound added for 8 hours then replaced with regular GM for 40 hours. The 24 + 24 group had GM + compound changed every 24 hrs. Finally, the 48-hr group had GM + compound on for the whole duration. Cells were exposed to MTT solution then measured on a spectrophotometer at 590 nM. All data

is represented by means with SEM (n = 8). * indicates significantly different than 5uM at 48hr within the same group.

CBD and CBN can increase the proliferation of myoblasts

Analysis using an MTT assay revealed supplementation with CBD at 1uM increased the proliferation of C2C12s in comparison to MTH (vehicle control) 1uM (p <0.0001). In addition, CBD at 5uM showed the same effect by significantly increasing proliferation compared to MTH at 5uM (p <0.0001). No significant differences were shown between CBD at 0.1uM and the vehicle control MTH at 0.1uM (p>0.05) (n=12) **(Figure 2A).**

CBN at 1uM increased the proliferative ability of myoblasts compared to CBN at 0.1uM**.** (p < 0.0005). Similarly, CBN at 5uM was able to increase proliferation compared to both CBN at 0.1uM and 1uM (p<0.0001, p <0.05), as well as MTH at 5uM (p<0.0001). At 0.1uM concentration, CBN and MTH showed no significant differences. Similarly, CBN at 1uM and MTH at 1uM showed no differences (p>0.05) (n=12) **(Figure 2B).**

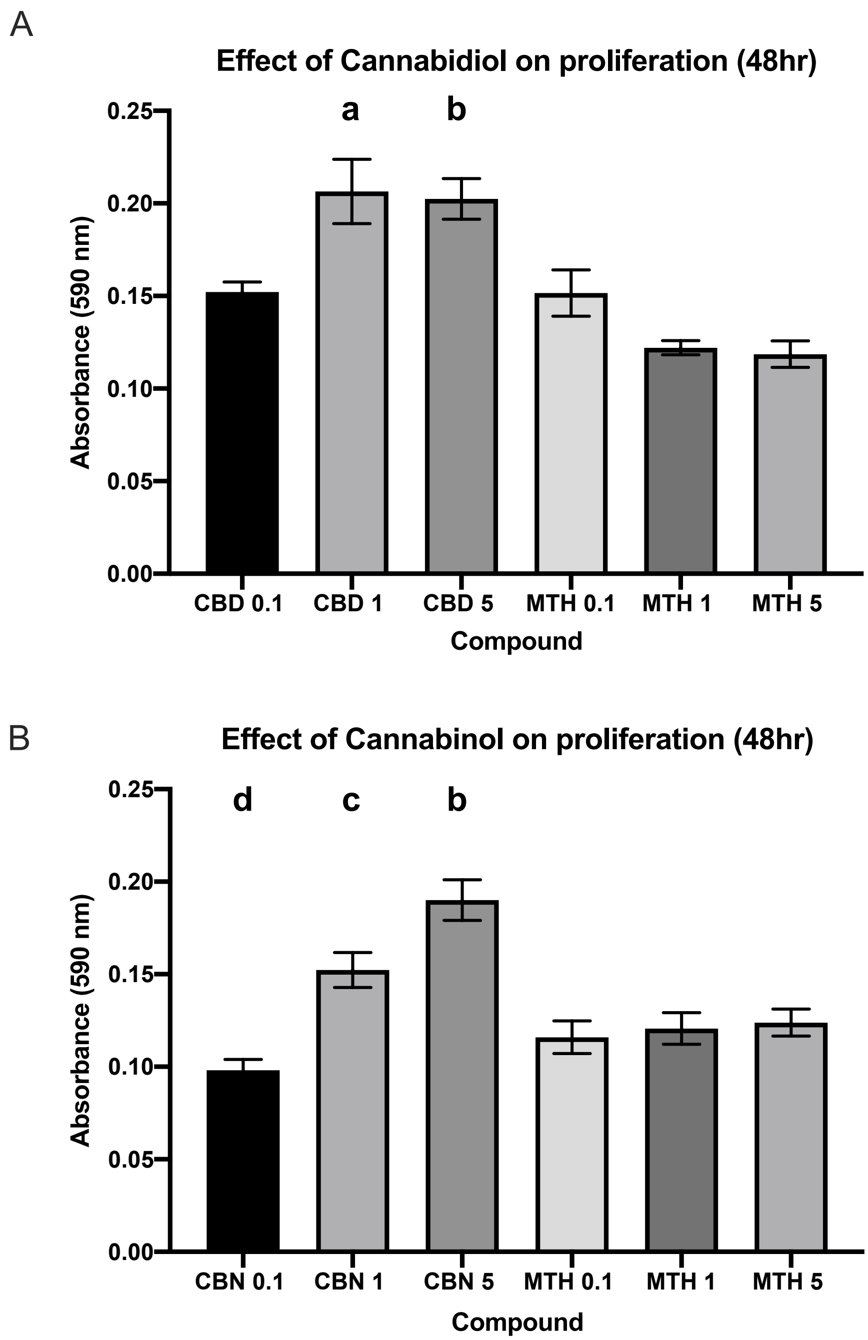


Figure 2. Proliferation of C2C12 myoblasts supplemented with their respective compounds (CBD, CBN, MTH) and concentrations (0.1, 1, 5uM) for 48hours. To quantify this experiment the MTT solution was applied to cells on a 96 well plate and measured using a spectrophotometer at 590nM. All graphs represented as a mean with SEM, data was considered significantly different when *P* was < 0.05 (n=12). 'a' indicates significantly different than MTH 1, 'b' indicates significantly different than MTH 5, 'c' indicates

significantly different than CBN 5, 'd' indicates significantly different than CBN 1 and CBN 5

No effect of compound supplementation on gene expression in myoblasts.

After 48 hrs of cell proliferation, mRNA expression of genes associated with the hypertrophic response were measured. No significant changes in Myf5, MyoD, Myogenin, FSTN and MSTN gene expression in C2C12 myoblasts were observed when supplemented with CBD, CBN or MTH at 5uM ($p>0.05$) ($n=6$) **(Figure 3A, B, C, D, E).**

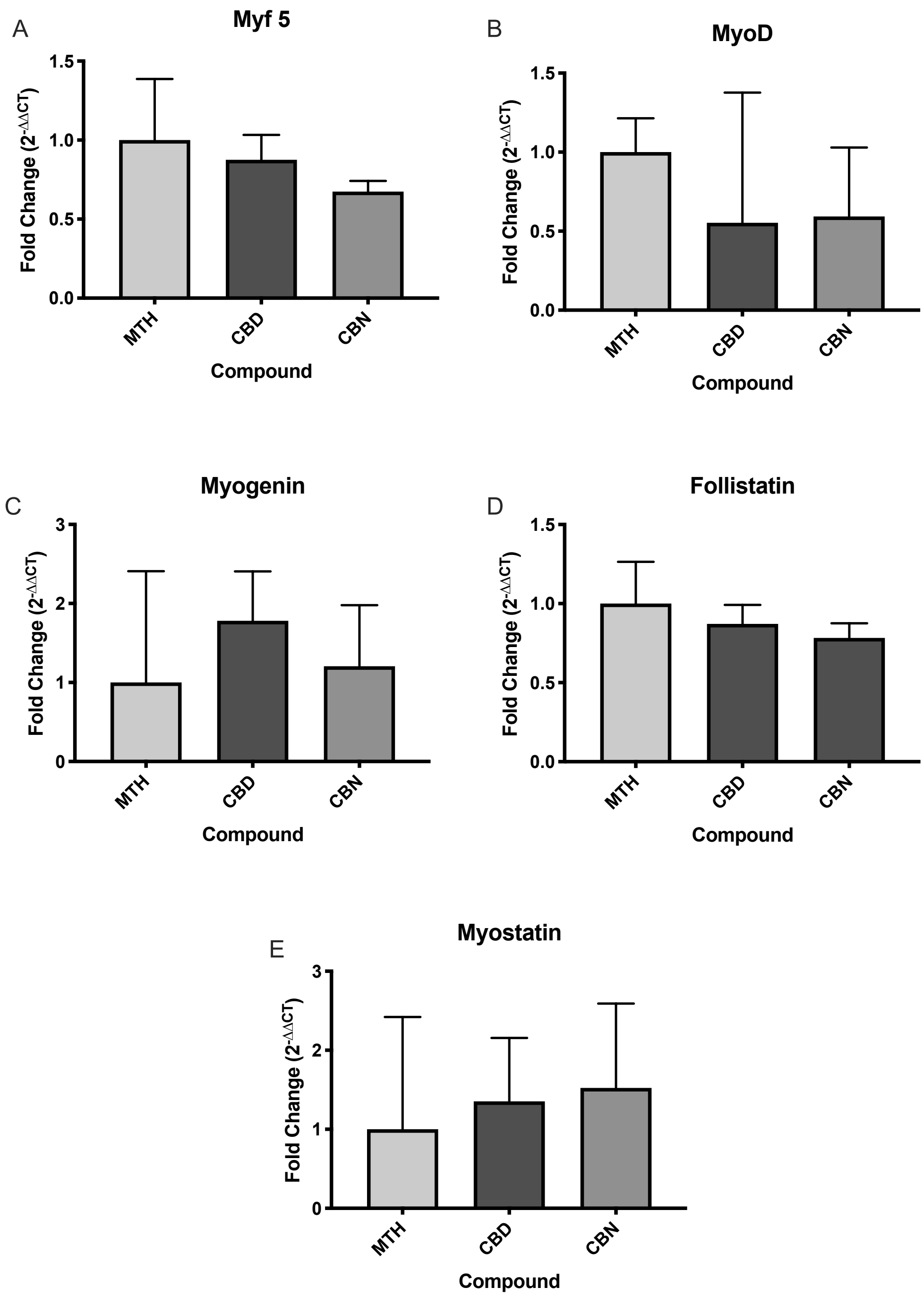


Figure 3. Fold change from MTH of gene expression during proliferation. GM + compound was administered at 40% cell confluence; with media being changed every 24hrs. RNA isolation and reverse transcription-PCR techniques were applied after the 2nd day. To quantify the amplification signal of gene expression, SYBR green assay was used in

combination with specific primers. Fold change was calculated by $2^{-(\Delta\Delta CT)}$. All graphs are shown as means with SEM (n=6).

No effect of compound supplementation on gene expression in myotubes.

Genes involved with signaling muscle growth were measured using qPCR in C2C12 myotubes that were differentiating for 7 days. DM was supplemented with CBD, CBN or MTH at 5uM. We report no increases to Myf5, MyoD, Myogenin, Follistatin and Myostatin gene expression after supplementation with CBD or CBN at 5uM ($p>0.05$) (n=6) **(Figure 4 A, B, C, D, E).**

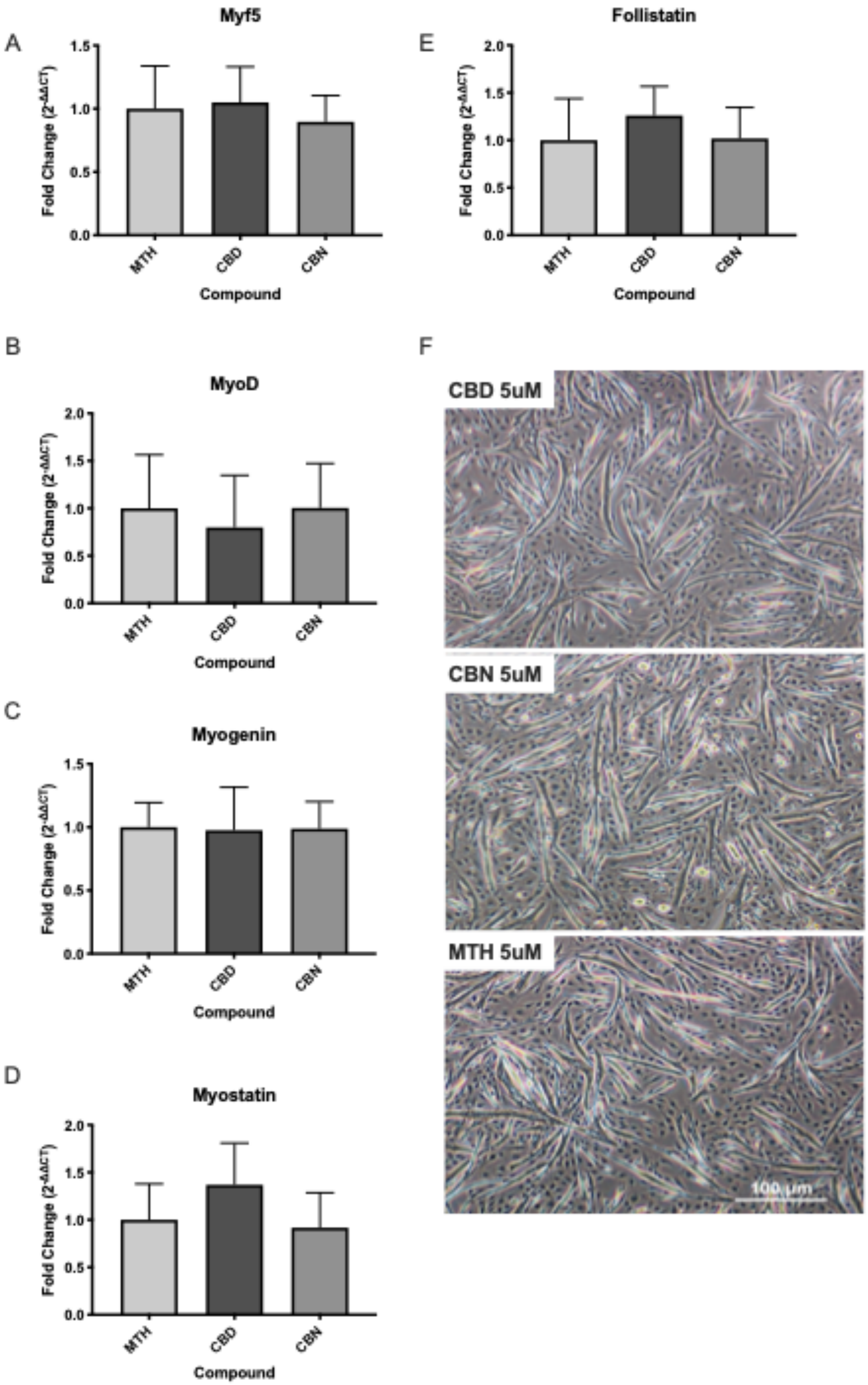

A
Myf5
Fold Change (2-ΔΔCT)
1.5
1.0
0.5
0.0
MTH
CBD
CBN
Compound
E
Follistatin
Fold Change (2-ΔΔCT)
2.0
1.5
1.0
0.5
0.0
MTH
CBD
CBN
Compound
B
MyoD
Fold Change (2-ΔΔCT)
2.0
1.5
1.0
0.5
0.0
MTH
CBD
CBN
Compound
C
Myogenin
Fold Change (2-ΔΔCT)
1.5
1.0
0.5
0.0
MTH
CBD
CBN
Compound
D
Myostatin
Fold Change (2-ΔΔCT)
2.0
1.5
1.0
0.5
0.0
MTH
CBD
CBN
Compound
F
CBD 5uM
CBN 5uM
MTH 5uM
100 µm

Figure 4. Fold change compared to MTH of gene expression during differentiation. Media was changed to DM when cells reached 80% confluency, then, DM + compound was added 24 hrs after at 5uM concentration. Media was changed every 48 hrs until the day 7 (A) Myf5 gene expression (B) MyoD gene expression (C) Myogenin gene expression (D) FSTN gene expression (E) MSTN gene expressions (F) Light microscopy image at 4x magnification of myotubes day 7 of differentiation. All graphs are shown in means with SEM (n=6).

CBD and CBN supplementation do not affect myotube diameter, surface area, or myonuclear index

Using myotube diameter, surface area and myonuclear index as indicators of myotube differentiation, it was determined that no significant increases were seen after supplementation with compounds at a concentration of 5 uM in any of the groups compared to the MTH group ($p>0.05$) ($n=4$) **(Figure 5 A, B, C).** Similarly, no significant increases were indicated after supplementation with a 1 uM concentration compared to the MTH group ($p>0.05$) ($n=3$) **(Figure 6 A, B, C)**

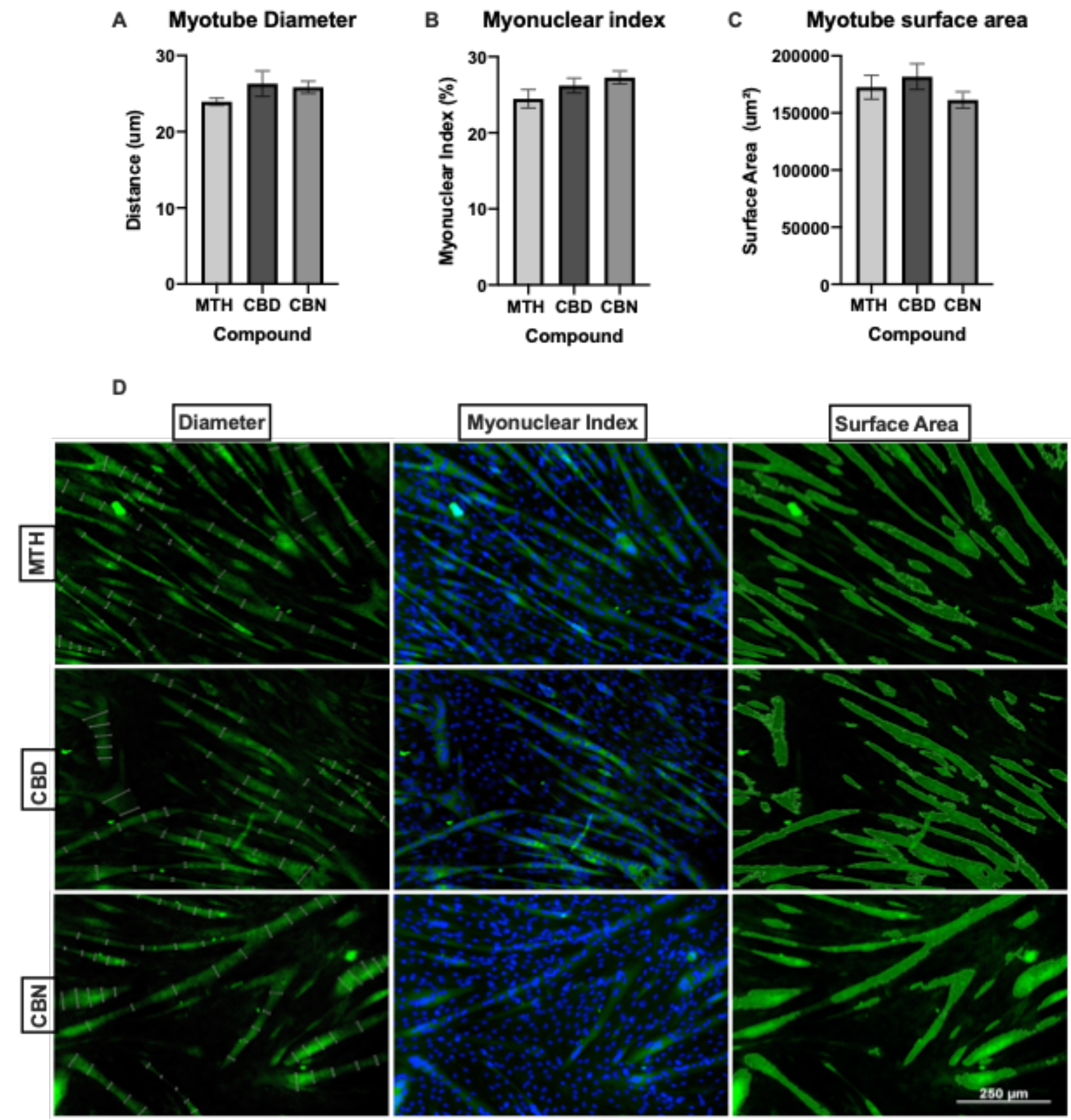


Figure 5. Data represents quantification of C2C12 differentiation after supplementation with CBD, CBN or MTH at 5uM in DM. Immunofluorescent protocols were used to stain for MHC I (green) and nuclei (blue). The analysis of each image was done on the Nikon Elements application. (A) Calculation of myotube diameter measured in micrometers (B) Myonuclear Index values are expressed in percentages. (C) Myotube surface area was quantified using the binary thresholding application for overall MHCI in uM^2. (D) Immunofluorescent images acquired from the NIKON Eclipse Ti microscope at 10x

magnification on the FITC and DAPI channel. All data represented as means with SEM (n=4).

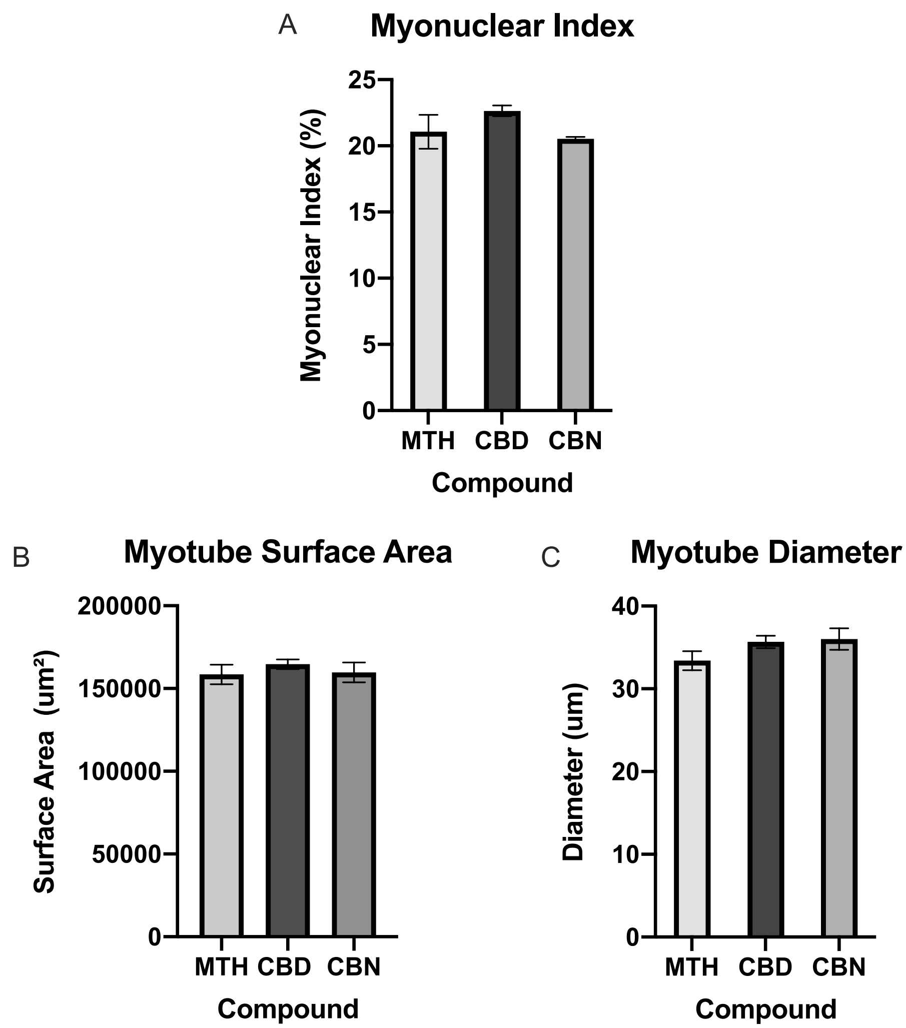


Figure 6. Quantification of C2C12 differentiation after supplementation of CBD, CBN or MTH at 1uM with DM. Immunofluorescent protocols were used to stain for MHC I and nuclei. The analysis of each image was done on the Nikon Elements application. (A) Calculation of myotube diameter measured in micrometers (B) Myonuclear Index values are expressed in percentages and are calculated by dividing number of nuclei in myotubes by the total nuclei in image then multiplying by 100. (C) Myotube surface area was quantified using the binary thresholding application for overall MHCI in uM^2. All data represented as means with SEM (n=3).

Significant decrease in follistatin gene expression following differentiation of myoblasts treated with CBD at 1 uM.

In comparison to CBN and MTH, CBD was able to decrease the gene expression of FSTN ($p<0.001$). Other analyses of gene expression showed no differences in Myf5, MyoD, Myogenin and MSTN in differentiated C2C12s ($p>0.05$) ($n=6$) **(Figure 7 A, B, C, D, E).**

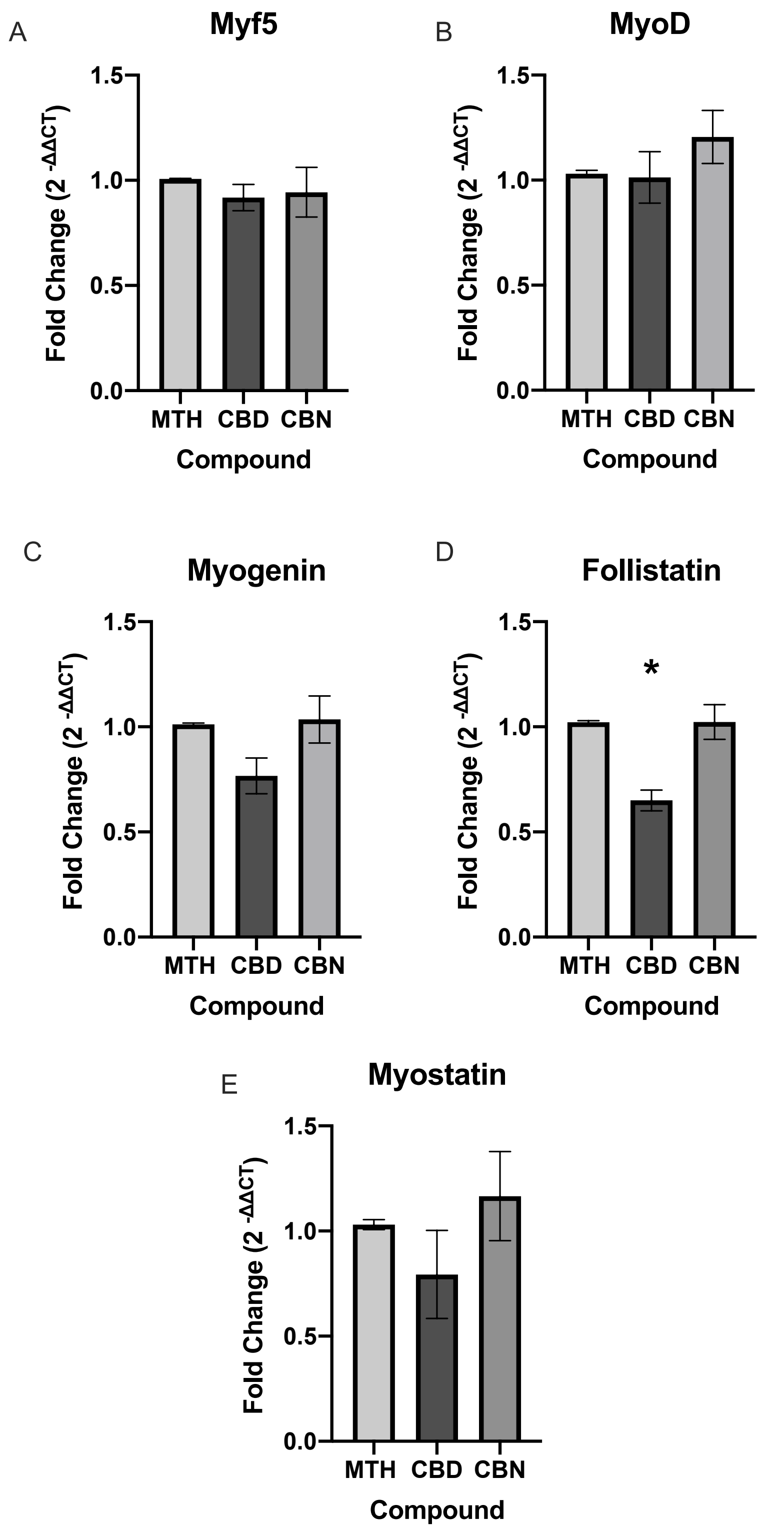

A
Myf5
B
MyoD
C
Myogenin
D
Follistatin
E
Myostatin
Fold Change (2 -ΔΔCT)
1.5
1.0
0.5
0.0
MTH CBD CBN
Compound
*

Figure 7. Fold change from MTH of gene expression during differentiation. DM was administered after 80% cell confluence, then, DM + compound was added 24 hrs after at 1uM concentration. Media was changed every 48 hrs until day 7. All graphs are shown in means with standard error means (n=6). * Indicates significantly different than CBN and MTH.

DISCUSSION

In the current investigation, we report enhanced proliferation of C2C12 cells following 48 hours of supplementation with CBD or CBN. It was also determined that replenishing growth media (GM) and compound every 24 hrs resulted in a greater proliferation of cells compared to not replenishing GM and compound for 48 hrs. Furthermore, since cell proliferation was augmented, it was hypothesized that gene expression of Myf5 and MyoD – the transcription factors known to initiate the myogenic regulatory response[22, 23, 24] – would be significantly higher in the presence of CBD and CBN. However, no notable changes in Myf5 or MyoD gene expression was observed, as was true for myogenin, FSTN and MSTN after supplementation with CBD or CBN at 5 uM. Likewise, no change was observed in gene expression of Myf5, MyoD, Myogenin, FSTN and MSTN in differentiating cells treated with either CBD or CBN at 5 uM. Using immunocytochemical analysis, we quantified the myotube surface area, diameter, and myonuclear index following differentiation, and report no notable differences between cells treated with CBD or CBN as compared to vehicle control-treated cells. To further evaluate the differentiation response to these compounds, a 1uM concentration was used in relation to the significant findings reported by Iannotti and colleagues (2018). Our results indicated that there were no considerable changes in myotube size, diameter, or myonuclear index. The gene expression of Myf5, MyoD, Myogenin, and MSTN were also unchanged, however, supplementation of CBD resulted in significantly lower gene expression of FSTN compared to CBN and MTH (1uM). Overall, our findings were not consistent with our hypothesis stated at the outset.

Based on previous literature[72,73, 146], we predicted that the supplementation of CBD and CBN would augment myotube formation and the transcription of myogenin, in addition to enhancing cell proliferation with a concomitant up-regulation of Myf5 and MyoD. Iannotti and colleagues (2018) found that CBD at a 1 uM concentration induced greater myotube formation and mRNA expression of myogenin in C2C12 myoblasts[73]. Notably, they also reported that a 3 uM concentration resulted in decreased myogenin transcription[73]. Results in the current study do no align with those previously published. We found no significant increase in myogenin expression or myotube formation at both 1 and 5 uM concentrations. The disparate findings are likely accounted for by the incubation time of CBD on cells. Iannotti and colleagues (2018) added cannabinoids to the DM for 5, 15 mins or 3 hrs, and then replaced it with regular DM for 72 hrs[73]. In comparison, we incubated differentiating cells with CBD for seven days, with media being changed every 48 hrs. The exposure time of CBD was significantly different, which resulted in examining changes in myotubes at different periods of growth. Moreover, it is unknown whether the same CBD compound was used between the two investigations. In the present study, the chemical structure and molecular weight of the compound are clear[139], whereas this information was not provided in the published study by Iannotti and colleagues (2018). Using the same compound is an important consideration since the agent can be derived synthetically or isolated from plants.

To determine the influence of CBD and CBN on the myogenic response, we investigated the gene expression of critical regulators of SC proliferation and differentiation[29, 49, 114, 138]. It has been well documented that MRFs orchestrate the myogenic program, and ultimately contribute to the growth, repair, and regeneration of muscle[138]. In the resting state, SCs are quiescent and can be identified by the expression of Pax7[20]. Up-regulation of Myf5 and MyoD, usually by exercise or injury, results in the activation of SC and subsequent entry into the cell cycle. SCs proliferate – referred to as myoblasts – and either self-renew or fuse with existing myofibers *in vivo*, or with other myoblasts *in vitro*. The expression of Mrf4 and myogenin commits myoblasts to differentiation[22, 23, 24], resulting in the repair and regeneration of muscle. Collectively, these transcription factors are important measures to determine whether CBD or CBN might influence myogenesis or growth of skeletal muscle

Other proteins implicated in the hypertrophic response include MSTN and FSTN. MSTN is a member of the TGB-β superfamily and is well known to negatively regulate muscle growth[29, 49, 114, 120]. MSTN is likely the most potent known regulator of muscle growth, whereby reducing the mRNA expression of MSTN by less than 1% in mice resulted in a 25% increase in muscle mass within three months[30]. Other studies have reported a similar phenomenon in humans, cattle, mice, sheep, and dogs through random mutations in the MSTN gene[32, 33, 34, 35, 36]. This has led to the hypothesis that inhibiting MSTN gene expression could potentially improve human performance or serve as a pharmaceutical target for treating muscle-related diseases, by increasing muscle mass[38, 121]. Indeed, the association between MSTN and sarcopenia has been shown in numerous studies, with results indicating the endogenous levels of MSTN may influence the prevalence of sarcopenia[37, 38]. McKay and colleagues (2012) demonstrated that MSTN protein is localized to SCs[44]. Based on their findings, it was determined that increased colocalization of MSTN in SC induced impairments in the myogenic capacity of aged muscle[44]. In another study, it was noted that inhibiting MSTN gene expression resulted in enhanced muscle growth in mice experiencing an age-related decline of muscle mass[49]. Given the unfavorable effect of increasing MSTN in old age, the delivery of FSTN presents a promising approach for increasing skeletal muscle mass. Specifically, FSTN is a robust antagonist of MSTN that has the primary function of binding and neutralizing members of the TGF-β superfamily[121]. The experimental overexpression of FSTN in mice significantly increases muscle mass while decreasing fat accumulation, compared to their wild type counterparts[49]. Therefore, it would be beneficial if a nutritional supplement was capable of inhibiting the expression of MSTN while increasing FSTN. In search of a novel compound that could target both proteins, Gutierrez-Salmean and colleagues (2014) investigated the influence of epicatechin – a polyphenol found in plants – in mice and humans[153]. It was determined that epicatechin decreased MSTN and β-galactosidase expression, as well as increased levels of genetic markers associated with myogenesis in mice[153]. As a proof of principle, it was also demonstrated in humans that treatment for seven days with epicatechin improved handgrip strength and increased the plasma levels of FSTN and decreases MSTN levels[153]. This evidence reveals a promising strategy for the role of nutrition in regulating the hypertrophic response. To determine if cannabinoids influenced the regulation of MSTN and FSTN, the current investigation measured the gene expression of MSTN and FSTN in C2C12 myotubes after supplementation with CBD or CBN for seven days. The evidence in our study suggests that both CBD and CBN did not affect the regulation of MSTN and FSTN.

Limitations and Future Directions

In the current study, it was noted that CBD and CBN were able to increase the growth rate of cells using immunofluorescent quantification via MTT. However, the gene expression data does not support this finding. It was unusual that Myf5 and MyoD gene expression were not up-regulated following treatment with CBD or CBN. The MTT assay measures mitochondrial quantity as an indicator of cell growth, whereby the expression of purple formazan metabolized from yellow MTT suggests an increase in cell growth, however, it is possible that CBD or CBN improved the metabolic capacity of these cells instead. It would be beneficial to examine other mechanisms involved in the cell cycle that can further elucidate the proliferation of myoblasts supplemented with CBD or CBN. To further examine cell proliferation, numerous enzymes known as cyclin-dependent kinases (Cdk) actively regulate the cell cycle when bound to cyclins[151]. In eukaryotic cells, these involve complex combinations through different phases of the cell cycle, which in turn provide additional control to the cell-cycle machinery[151]. In addition to Myf5 and MyoD, these regulatory proteins can also influence the initiation of the cell through the G1/ S/ G2/ and M phase, which can provide a clear picture of the events during cell growth. In consideration to our experimental timeline during cell exposure to CBD or CBN, the analysis of gene expression and myotube formation was performed after 48 hrs and seven days in proliferation and differentiation, respectively. In doing so, the possible impacts on myoblasts and myotubes may have been realized earlier on and the effects of the supplement may have diminished thereafter. Another unexpected outcome was the decreased expression of FSTN after supplementation with CBD at 1 uM, although carefully controlled for, this anomaly could stem from the usage of cells that were in a different passage or level of cell viability.

Evidence using a 2D culture model in drug discovery has identified limitations of *in vitro* cell responses to guide pharmaceutical interventions. The nature of cells grown in a monolayer does not resemble the complete structure of skeletal muscle[118]. Notably, the absence of an extracellular matrix (ECM) is a significant limitation to *in vitro* models. It was thought that the ECM simply provided structural support; however, it is now appreciated that the ECM is capable of influencing most aspects of cell behaviour[118]. Components of the ECM, include factors such as matrix proteins, glycoproteins, proteoglycans, and ECM sequestered growth factors[118]. In the absence of factors such as those listed above, a 2D *in vitro* approach is inherently limiting since many factors can influence cell differentiation and proliferation[118]. It has been suggested that an ideal culture model should incorporate tissue-specific stiffness, oxygen, nutrient and metabolic waste gradients, a combination of tissue-specific scaffolding and cell-to-cell and cell-to-ECM interactions[117]. However, to our knowledge, no current 3D culture method meets all of the criteria mentioned above. Rather each 3D culture model has its advantages, but also limitations. A feasible option for future investigations is to use a 2.5D model in which cells are plated on top of a thick layer of ECM. The method allows for a better representation of the complex microenvironment seen *in vivo*, while also being suitable for the current objectives in this project – providing high throughput screening, and tissue-specific differentiation in cells[117]. In conjunction with the proposed method, primary muscle cells could also be added as a means to better represent the physiological structures in muscle tissue. Furthermore, the cell-based assays used in the current investigation – such as MTT, immunocytochemistry, and qPCR – are still applicable in a 2.5D culture approach.

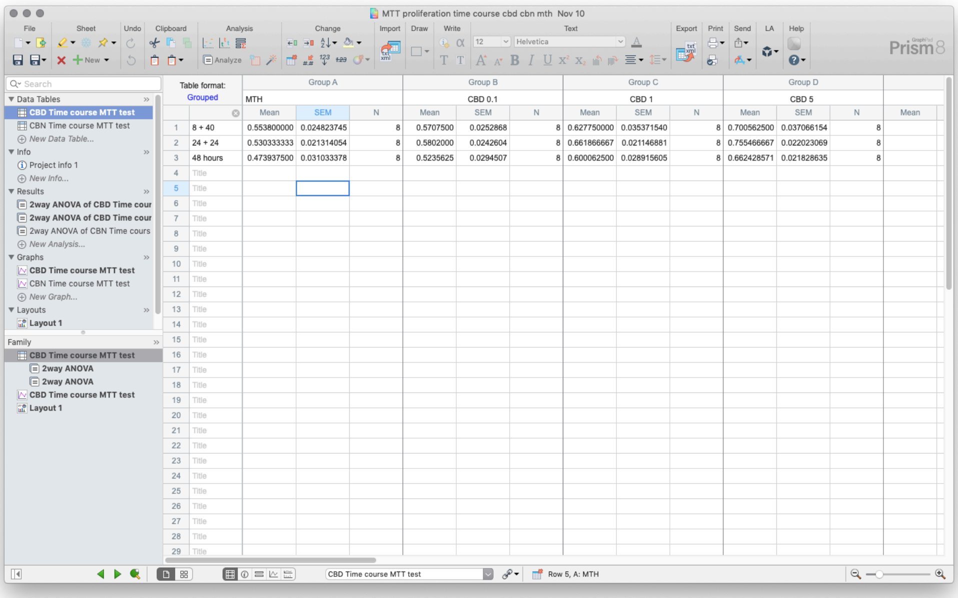
Conclusion

In summary, the primary objective of this investigation was to evaluate the effect of CBD and CBN supplementation on murine myoblast proliferation and differentiation, to determine whether these compounds might enhance the myogenic response. Based on our results, it was evident that following supplementation, both CBD and CBN improved cell proliferation after 48 hrs in culture but did not impact differentiation after seven days of incubation. Studies have shown the effectiveness of CBD and CBN as an anti-inflammatory and antioxidative medicine[123, 124, 125]. However, recent evidence from Giacoppo et al. 2016 and Iannotti et al. 2018 has suggested a potential for CBD as effective therapeutic interventions for muscular dystrophies. The fact that CBD and CBN might serve an as effective strategy for muscle-related pathology is not a trivial matter, given that the proportion of older adults in society is rapidly growing. Future directions for this research should aim to focus on evaluating the absorption, distribution, metabolism, and excretion of CBD and CBN *in vitro*. As previously mentioned, cell culture techniques involving HepG2 are an effective and commonly used method when measuring the cytotoxicity of new drugs and compounds[137]; because of the high degree of morphological and functional resemblance to the liver, they are a suitable model to study the metabolism of CBD and CBN supplementation, which would provide a basis for the safety and feasibility of consuming cannabinoids. To identify improvements in mitochondrial function from CBD or CBN, the Agilent Seahorse XF analyzer can be utilized to measure glycolysis and oxidative phosphorylation in cells, which will expand upon the findings shown here. Other future experiments should also evaluate the gene expression of cyclin-dependent kinases such as CDK1, which has been shown to be essential for cell proliferation[152], and will provide a complete picture of possible cell growth. Finally, with respect to our timeline for analysis of gene expression late into differentiation (day 7), it would be of interest to look at the acute response from exposure to supplements for both proliferating and differentiating cells (i.e. day 1, 5 and 7), potentially presenting a significant effect earlier on in the incubation period.

APPENDIX: SUPPLEMENTARY PICS, RAW DATA AND STATISTICS

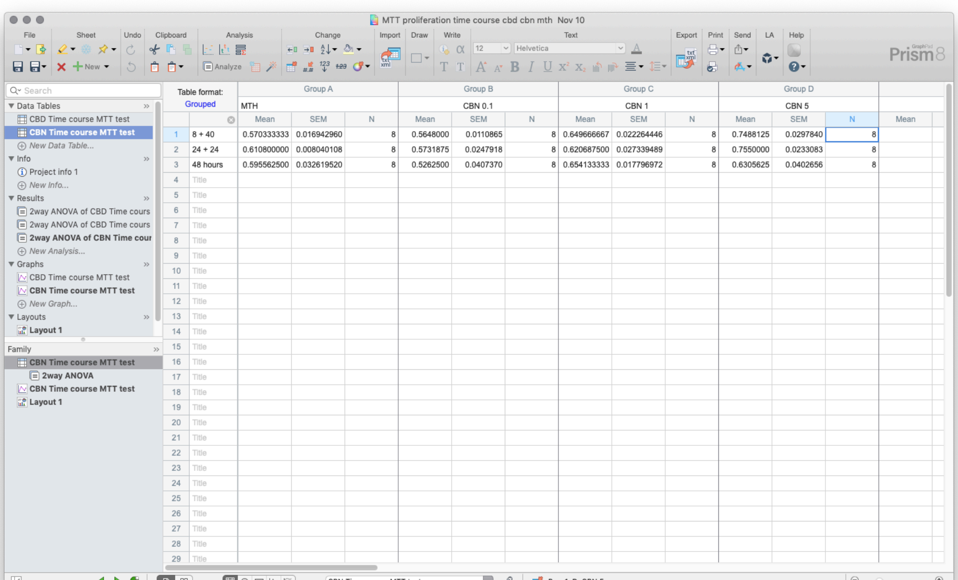
RAW DATA Proliferation Time Course Experiment (8+40hrs, 24+24hrs, 48hrs)

CBD



	Group A			Group B			Group C			Group D		
	MTH			CBD 0.1			CBD 1			CBD 5		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
8 + 40	0.553800000	0.024823745	8	0.5707500	0.0252868	8	0.627750000	0.035371540	8	0.700562500	0.037066154	8
24 + 24	0.530333333	0.021314054	8	0.5802000	0.0242604	8	0.661866667	0.021146881	8	0.755466667	0.022023069	8
48 hours	0.473937500	0.031033378	8	0.5235625	0.0294507	8	0.600062500	0.028915605	8	0.662428571	0.021828635	8

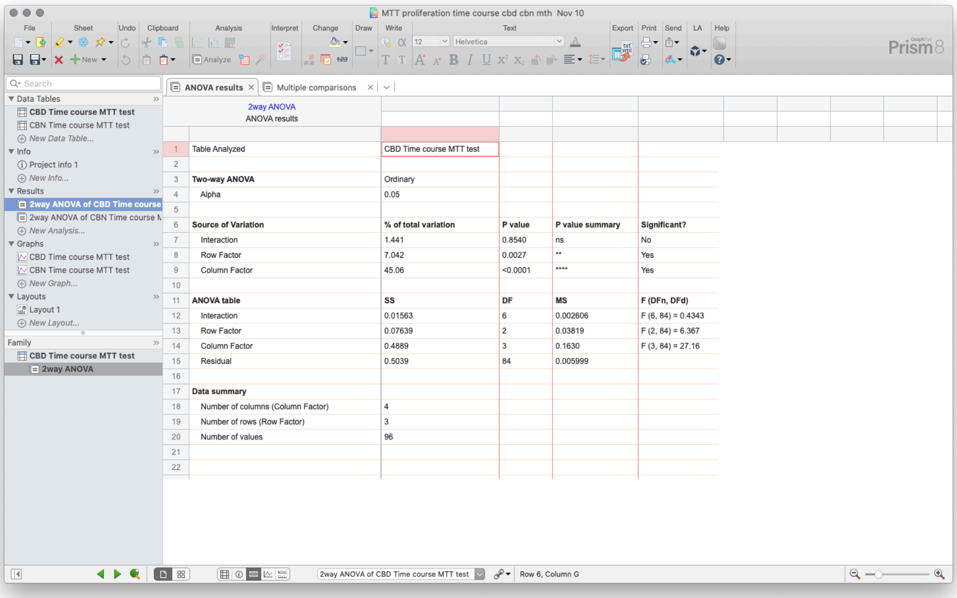
CBN



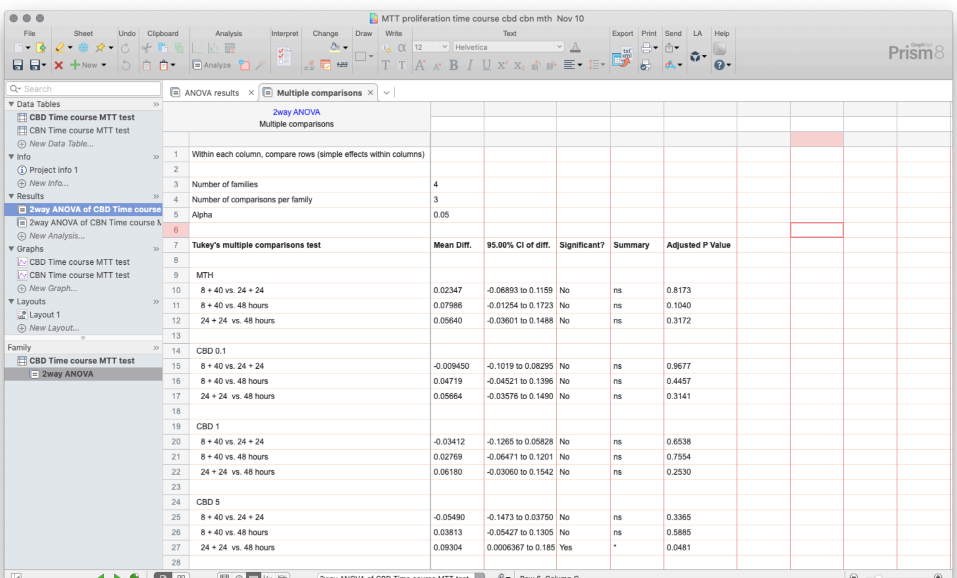
	Group A			Group B			Group C			Group D		
	MTH			CBN 0.1			CBN 1			CBN 5		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
8 + 40	0.570333333	0.016942960	8	0.5648000	0.0110865	8	0.649666667	0.022264446	8	0.7488125	0.0297840	8
24 + 24	0.610800000	0.008040108	8	0.5731875	0.0247918	8	0.620687500	0.027339489	8	0.7550000	0.0233083	8
48 hours	0.595562500	0.032619520	8	0.5262500	0.0407370	8	0.654133333	0.017796972	8	0.6305625	0.0402656	8

TWO-WAY ANOVA Analysis of Proliferation Time Course Results

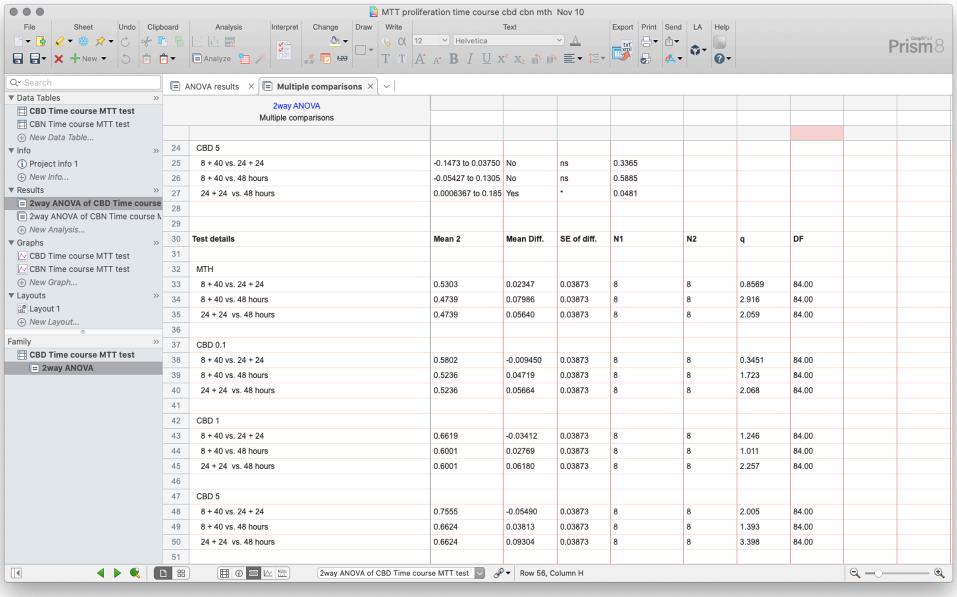
CBD



	2way ANOVA ANOVA results				
1	Table Analyzed	CBD Time course MTT test			
2					
3	**Two-way ANOVA**	Ordinary			
4	Alpha	0.05			
5					
6	**Source of Variation**	**% of total variation**	**P value**	**P value summary**	**Significant?**
7	Interaction	1.441	0.8540	ns	No
8	Row Factor	7.042	0.0027	**	Yes
9	Column Factor	45.06	<0.0001	****	Yes
10					
11	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**
12	Interaction	0.01563	6	0.002606	F (6, 84) = 0.4343
13	Row Factor	0.07639	2	0.03819	F (2, 84) = 6.367
14	Column Factor	0.4889	3	0.1630	F (3, 84) = 27.16
15	Residual	0.5039	84	0.005999	
16					
17	**Data summary**				
18	Number of columns (Column Factor)	4			
19	Number of rows (Row Factor)	3			
20	Number of values	96			
21					
22					



	2way ANOVA Multiple comparisons					
1	Within each column, compare rows (simple effects within columns)					
2						
3	Number of families	4				
4	Number of comparisons per family	3				
5	Alpha	0.05				
6						
7	**Tukey's multiple comparisons test**	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**
8						
9	MTH					
10	8 + 40 vs. 24 + 24	0.02347	-0.06893 to 0.1159	No	ns	0.8173
11	8 + 40 vs. 48 hours	0.07986	-0.01254 to 0.1723	No	ns	0.1040
12	24 + 24 vs. 48 hours	0.05640	-0.03601 to 0.1488	No	ns	0.3172
13						
14	CBD 0.1					
15	8 + 40 vs. 24 + 24	-0.009450	-0.1019 to 0.08295	No	ns	0.9677
16	8 + 40 vs. 48 hours	0.04719	-0.04521 to 0.1396	No	ns	0.4457
17	24 + 24 vs. 48 hours	0.05664	-0.03576 to 0.1490	No	ns	0.3141
18						
19	CBD 1					
20	8 + 40 vs. 24 + 24	-0.03412	-0.1265 to 0.05828	No	ns	0.6538
21	8 + 40 vs. 48 hours	0.02769	-0.06471 to 0.1201	No	ns	0.7554
22	24 + 24 vs. 48 hours	0.06180	-0.03060 to 0.1542	No	ns	0.2530
23						
24	CBD 5					
25	8 + 40 vs. 24 + 24	-0.05490	-0.1473 to 0.03750	No	ns	0.3365
26	8 + 40 vs. 48 hours	0.03813	-0.05427 to 0.1305	No	ns	0.5885
27	24 + 24 vs. 48 hours	0.09304	0.0006367 to 0.185	Yes	*	0.0481
28						

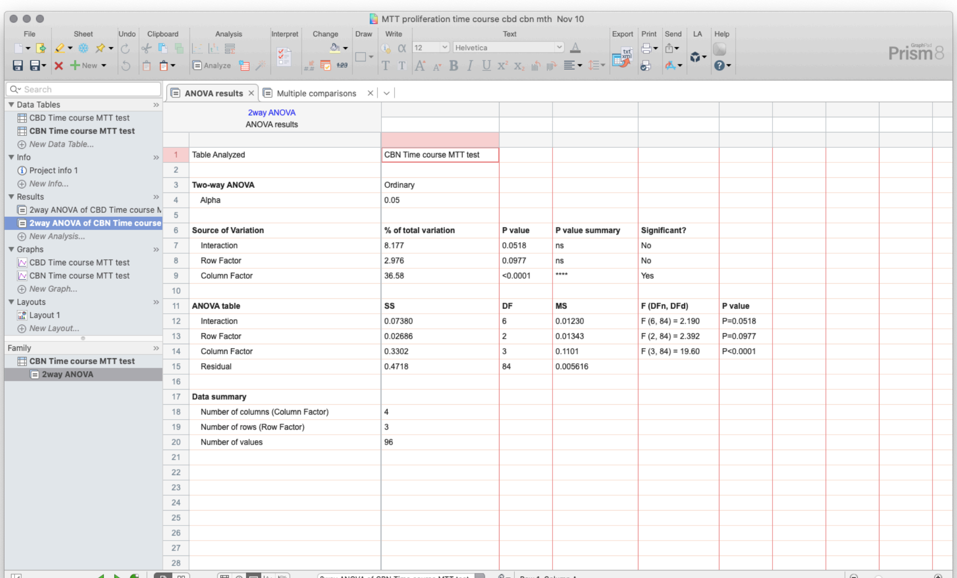


MTT proliferation time course cbd cbn mth Nov 10

2way ANOVA – Multiple comparisons

24	CBD 5							
25	8 + 40 vs. 24 + 24	-0.1473 to 0.03750	No	ns	0.3365			
26	8 + 40 vs. 48 hours	-0.05427 to 0.1305	No	ns	0.5885			
27	24 + 24 vs. 48 hours	0.0006367 to 0.185	Yes	*	0.0481			
28								
29								
30	**Test details**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**N1**	**N2**	**q**	**DF**
31								
32	MTH							
33	8 + 40 vs. 24 + 24	0.5303	0.02347	0.03873	8	8	0.8569	84.00
34	8 + 40 vs. 48 hours	0.4739	0.07986	0.03873	8	8	2.916	84.00
35	24 + 24 vs. 48 hours	0.4739	0.05640	0.03873	8	8	2.059	84.00
36								
37	CBD 0.1							
38	8 + 40 vs. 24 + 24	0.5802	-0.009450	0.03873	8	8	0.3451	84.00
39	8 + 40 vs. 48 hours	0.5236	0.04719	0.03873	8	8	1.723	84.00
40	24 + 24 vs. 48 hours	0.5236	0.05664	0.03873	8	8	2.068	84.00
41								
42	CBD 1							
43	8 + 40 vs. 24 + 24	0.6619	-0.03412	0.03873	8	8	1.246	84.00
44	8 + 40 vs. 48 hours	0.6001	0.02769	0.03873	8	8	1.011	84.00
45	24 + 24 vs. 48 hours	0.6001	0.06180	0.03873	8	8	2.257	84.00
46								
47	CBD 5							
48	8 + 40 vs. 24 + 24	0.7555	-0.05490	0.03873	8	8	2.005	84.00
49	8 + 40 vs. 48 hours	0.6624	0.03813	0.03873	8	8	1.393	84.00
50	24 + 24 vs. 48 hours	0.6624	0.09304	0.03873	8	8	3.398	84.00
51								

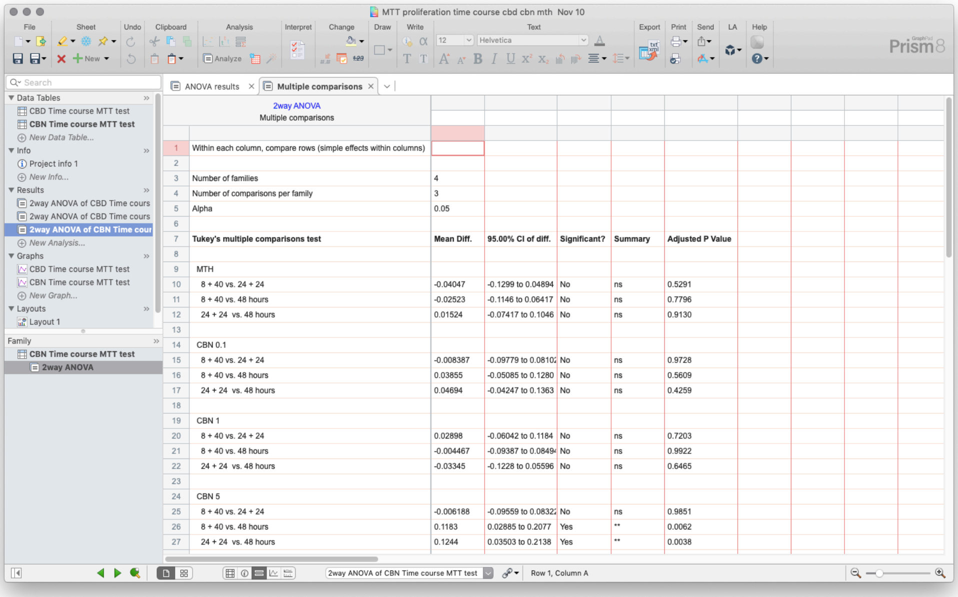
CBN



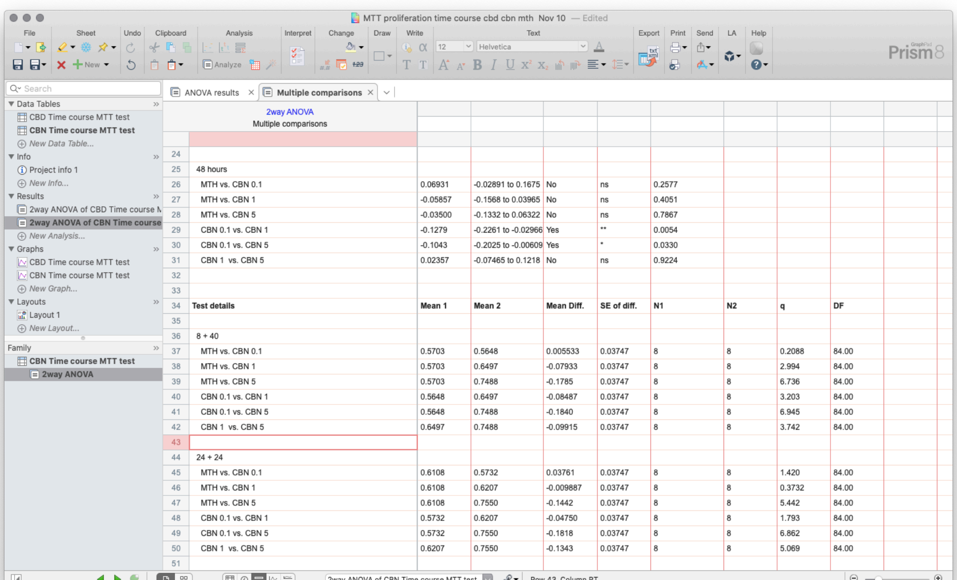
MTT proliferation time course cbd cbn mth Nov 10

2way ANOVA – ANOVA results

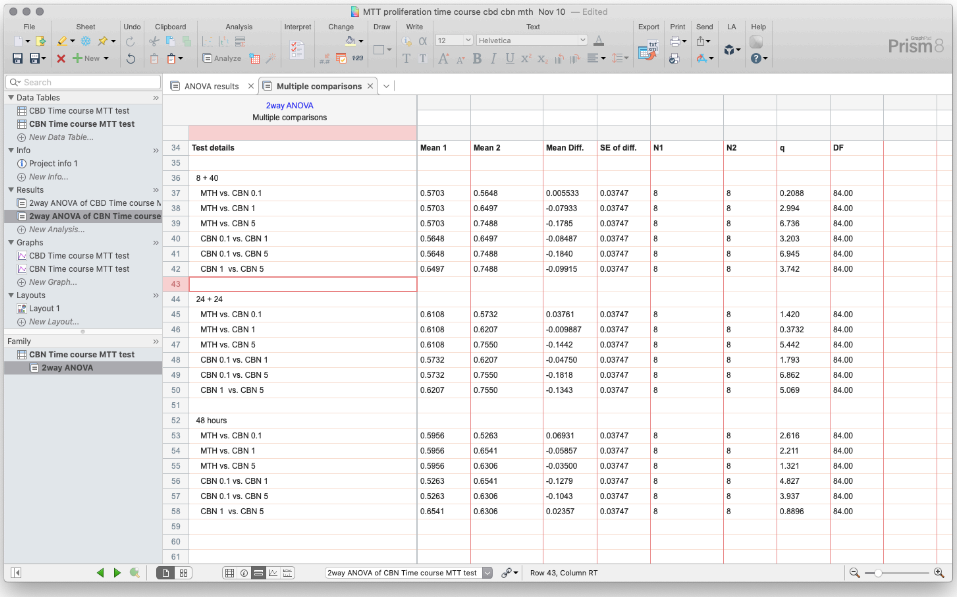
1	Table Analyzed	CBN Time course MTT test				
2						
3	**Two-way ANOVA**	Ordinary				
4	Alpha	0.05				
5						
6	**Source of Variation**	**% of total variation**	**P value**	**P value summary**	**Significant?**	
7	Interaction	8.177	0.0518	ns	No	
8	Row Factor	2.976	0.0977	ns	No	
9	Column Factor	36.58	<0.0001	****	Yes	
10						
11	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
12	Interaction	0.07380	6	0.01230	F (6, 84) = 2.190	P=0.0518
13	Row Factor	0.02686	2	0.01343	F (2, 84) = 2.392	P=0.0977
14	Column Factor	0.3302	3	0.1101	F (3, 84) = 19.60	P<0.0001
15	Residual	0.4718	84	0.005616		
16						
17	**Data summary**					
18	Number of columns (Column Factor)	4				
19	Number of rows (Row Factor)	3				
20	Number of values	96				



	2way ANOVA Multiple comparisons					
1	Within each column, compare rows (simple effects within columns)					
2						
3	Number of families	4				
4	Number of comparisons per family	3				
5	Alpha	0.05				
6						
7	**Tukey's multiple comparisons test**	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**
8						
9	MTH					
10	8 + 40 vs. 24 + 24	-0.04047	-0.1299 to 0.04894	No	ns	0.5291
11	8 + 40 vs. 48 hours	-0.02523	-0.1146 to 0.06417	No	ns	0.7796
12	24 + 24 vs. 48 hours	0.01524	-0.07417 to 0.1046	No	ns	0.9130
13						
14	CBN 0.1					
15	8 + 40 vs. 24 + 24	-0.008387	-0.09779 to 0.08102	No	ns	0.9728
16	8 + 40 vs. 48 hours	0.03855	-0.05085 to 0.1280	No	ns	0.5609
17	24 + 24 vs. 48 hours	0.04694	-0.04247 to 0.1363	No	ns	0.4259
18						
19	CBN 1					
20	8 + 40 vs. 24 + 24	0.02898	-0.06042 to 0.1184	No	ns	0.7203
21	8 + 40 vs. 48 hours	-0.004467	-0.09387 to 0.08494	No	ns	0.9922
22	24 + 24 vs. 48 hours	-0.03345	-0.1228 to 0.05596	No	ns	0.6465
23						
24	CBN 5					
25	8 + 40 vs. 24 + 24	-0.006188	-0.09559 to 0.08322	No	ns	0.9851
26	8 + 40 vs. 48 hours	0.1183	0.02885 to 0.2077	Yes	**	0.0062
27	24 + 24 vs. 48 hours	0.1244	0.03503 to 0.2138	Yes	**	0.0038



	2way ANOVA Multiple comparisons								
24									
25	48 hours								
26	MTH vs. CBN 0.1	0.06931	-0.02891 to 0.1675	No	ns	0.2577			
27	MTH vs. CBN 1	-0.05857	-0.1568 to 0.03965	No	ns	0.4051			
28	MTH vs. CBN 5	-0.03500	-0.1332 to 0.06322	No	ns	0.7867			
29	CBN 0.1 vs. CBN 1	-0.1279	-0.2261 to -0.02966	Yes	**	0.0054			
30	CBN 0.1 vs. CBN 5	-0.1043	-0.2025 to -0.00609	Yes	*	0.0330			
31	CBN 1 vs. CBN 5	0.02357	-0.07465 to 0.1218	No	ns	0.9224			
32									
33									
34	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**N1**	**N2**	**q**	**DF**
35									
36	8 + 40								
37	MTH vs. CBN 0.1	0.5703	0.5648	0.005533	0.03747	8	8	0.2088	84.00
38	MTH vs. CBN 1	0.5703	0.6497	-0.07933	0.03747	8	8	2.994	84.00
39	MTH vs. CBN 5	0.5703	0.7488	-0.1785	0.03747	8	8	6.736	84.00
40	CBN 0.1 vs. CBN 1	0.5648	0.6497	-0.08487	0.03747	8	8	3.203	84.00
41	CBN 0.1 vs. CBN 5	0.5648	0.7488	-0.1840	0.03747	8	8	6.945	84.00
42	CBN 1 vs. CBN 5	0.6497	0.7488	-0.09915	0.03747	8	8	3.742	84.00
43									
44	24 + 24								
45	MTH vs. CBN 0.1	0.6108	0.5732	0.03761	0.03747	8	8	1.420	84.00
46	MTH vs. CBN 1	0.6108	0.6207	-0.009887	0.03747	8	8	0.3732	84.00
47	MTH vs. CBN 5	0.6108	0.7550	-0.1442	0.03747	8	8	5.442	84.00
48	CBN 0.1 vs. CBN 1	0.5732	0.6207	-0.04750	0.03747	8	8	1.793	84.00
49	CBN 0.1 vs. CBN 5	0.5732	0.7550	-0.1818	0.03747	8	8	6.862	84.00
50	CBN 1 vs. CBN 5	0.6207	0.7550	-0.1343	0.03747	8	8	5.069	84.00
51									



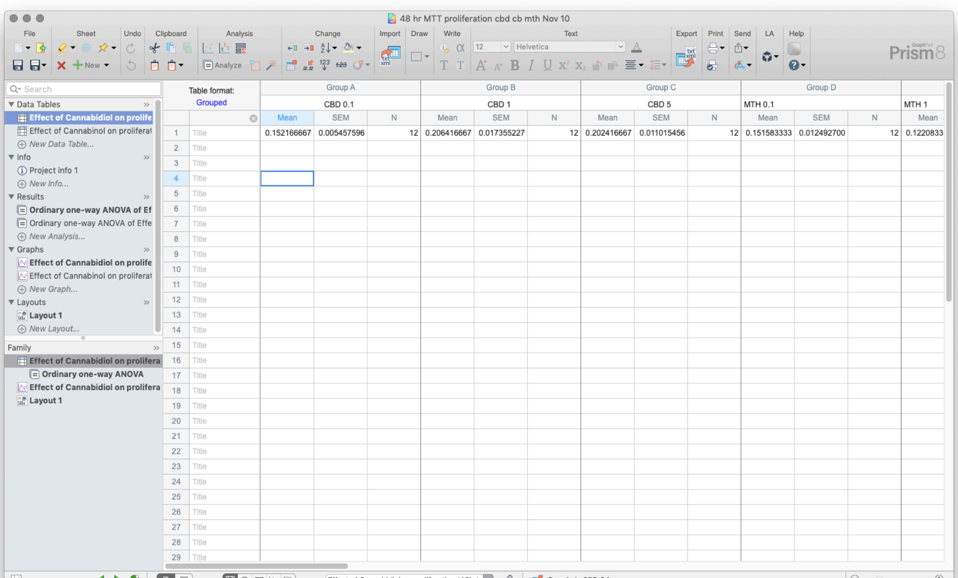
MTT proliferation time course cbd cbn mth Nov 10 — Edited

2way ANOVA
Multiple comparisons

	Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
35									
36	8 + 40								
37	MTH vs. CBN 0.1	0.5703	0.5648	0.005533	0.03747	8	8	0.2088	84.00
38	MTH vs. CBN 1	0.5703	0.6497	-0.07933	0.03747	8	8	2.994	84.00
39	MTH vs. CBN 5	0.5703	0.7488	-0.1785	0.03747	8	8	6.736	84.00
40	CBN 0.1 vs. CBN 1	0.5648	0.6497	-0.08487	0.03747	8	8	3.203	84.00
41	CBN 0.1 vs. CBN 5	0.5648	0.7488	-0.1840	0.03747	8	8	6.945	84.00
42	CBN 1 vs. CBN 5	0.6497	0.7488	-0.09915	0.03747	8	8	3.742	84.00
43									
44	24 + 24								
45	MTH vs. CBN 0.1	0.6108	0.5732	0.03761	0.03747	8	8	1.420	84.00
46	MTH vs. CBN 1	0.6108	0.6207	-0.009887	0.03747	8	8	0.3732	84.00
47	MTH vs. CBN 5	0.6108	0.7550	-0.1442	0.03747	8	8	5.442	84.00
48	CBN 0.1 vs. CBN 1	0.5732	0.6207	-0.04750	0.03747	8	8	1.793	84.00
49	CBN 0.1 vs. CBN 5	0.5732	0.7550	-0.1818	0.03747	8	8	6.862	84.00
50	CBN 1 vs. CBN 5	0.6207	0.7550	-0.1343	0.03747	8	8	5.069	84.00
51									
52	48 hours								
53	MTH vs. CBN 0.1	0.5956	0.5263	0.06931	0.03747	8	8	2.616	84.00
54	MTH vs. CBN 1	0.5956	0.6541	-0.05857	0.03747	8	8	2.211	84.00
55	MTH vs. CBN 5	0.5956	0.6306	-0.03500	0.03747	8	8	1.321	84.00
56	CBN 0.1 vs. CBN 1	0.5263	0.6541	-0.1279	0.03747	8	8	4.827	84.00
57	CBN 0.1 vs. CBN 5	0.5263	0.6306	-0.1043	0.03747	8	8	3.937	84.00
58	CBN 1 vs. CBN 5	0.6541	0.6306	0.02357	0.03747	8	8	0.8896	84.00

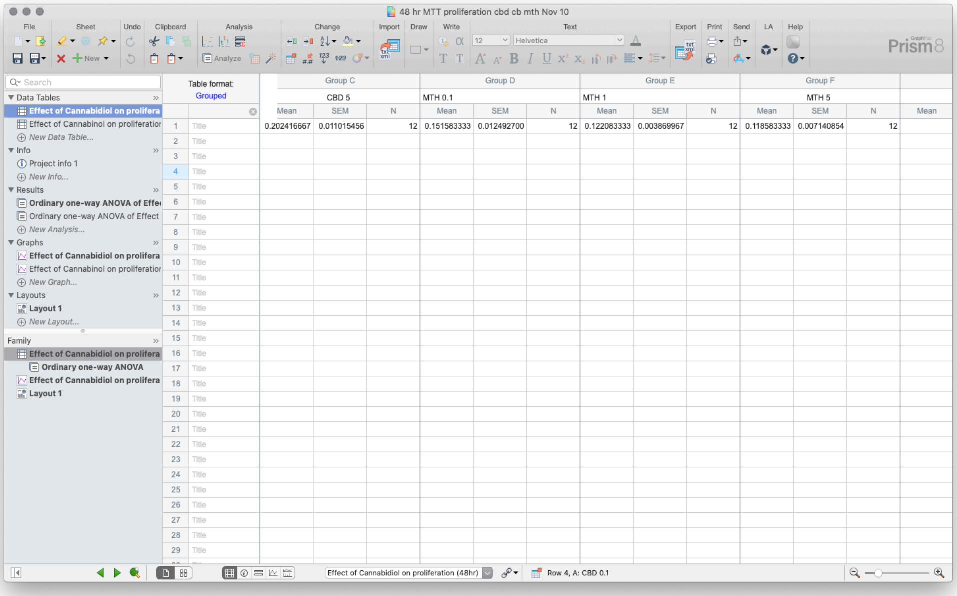
RAW DATA 48 hours Proliferation Concentration Experiment (0.1uM, 1uM, 5uM)

CBD

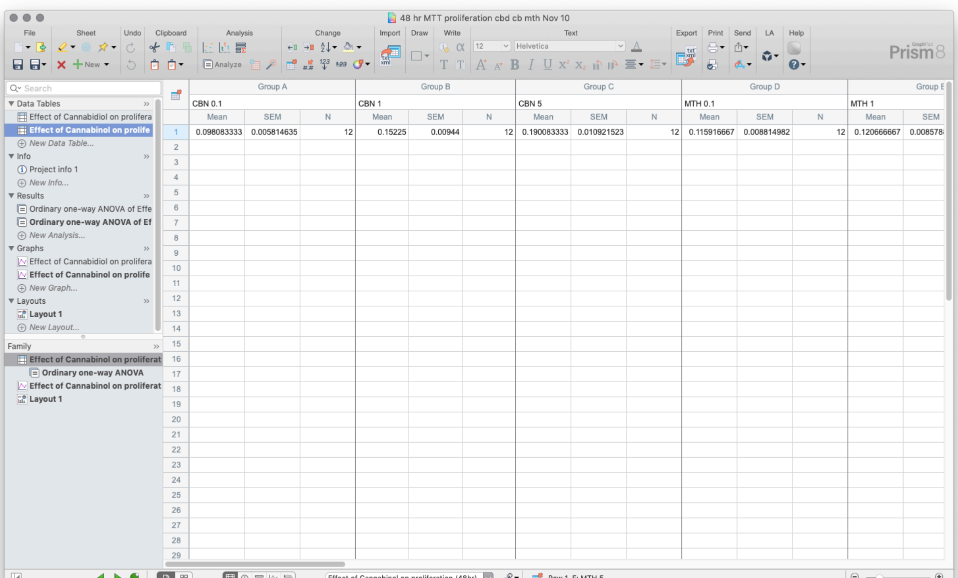


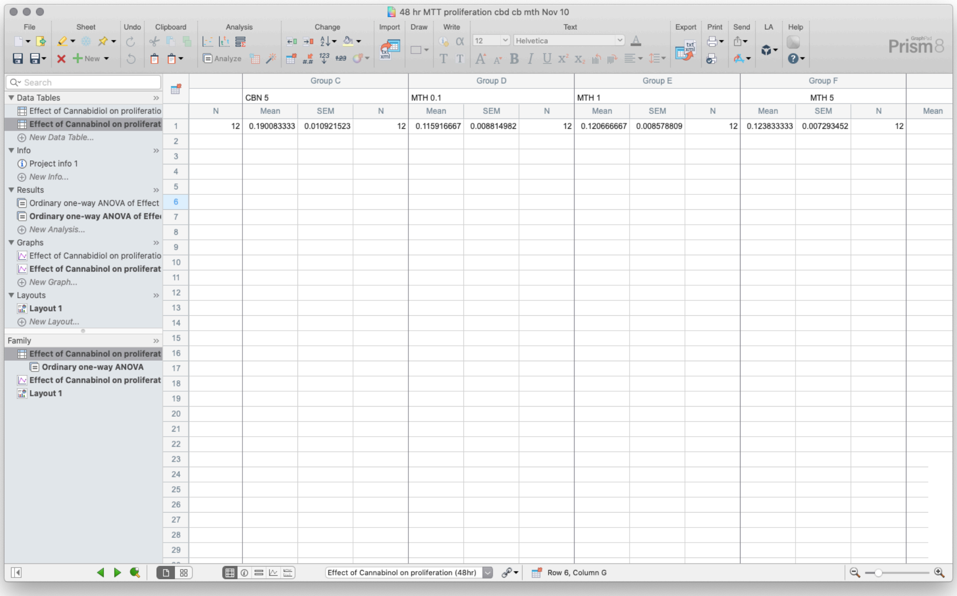
48 hr MTT proliferation cbd cb mth Nov 10

Table format: Grouped	Group A			Group B			Group C			Group D			
	CBD 0.1			CBD 1			CBD 5			MTH 0.1			MTH 1
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean
1 Title	0.152166667	0.005457596	12	0.206416667	0.017355227	12	0.202416667	0.011015456	12	0.151583333	0.012492700	12	0.1220833


48 hr MTT proliferation cbd cb mth Nov 10
Data Tables
Effect of Cannabidiol on prolifera
Effect of Cannabinol on proliferatio
Results
Ordinary one-way ANOVA of Effe
Ordinary one-way ANOVA of Effect
Graphs
Effect of Cannabidiol on prolifera
Effect of Cannabinol on proliferatio
Layouts
Layout 1
Table format:
Grouped
Group C
CBD 5
Group D
MTH 0.1
Group E
MTH 1
Group F
MTH 5
Mean
SEM
N
0.202416667
0.011015456
12
0.151583333
0.012492700
12
0.122083333
0.003869967
12
0.118583333
0.007140854
12
Effect of Cannabidiol on proliferation (48hr)
Row 4, A: CBD 0.1

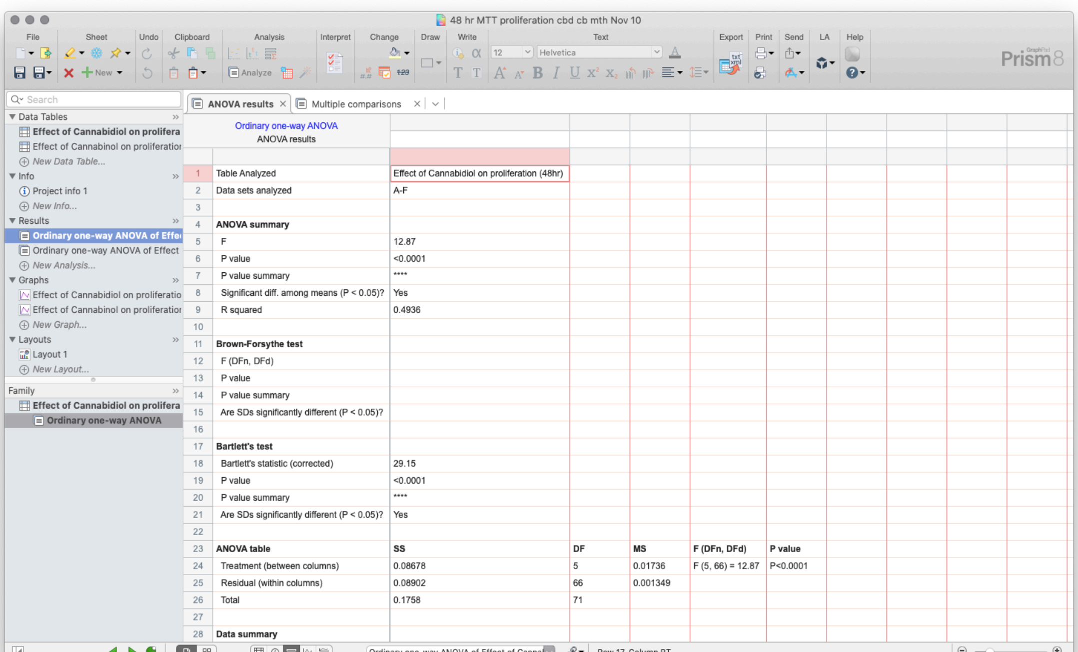
CBN

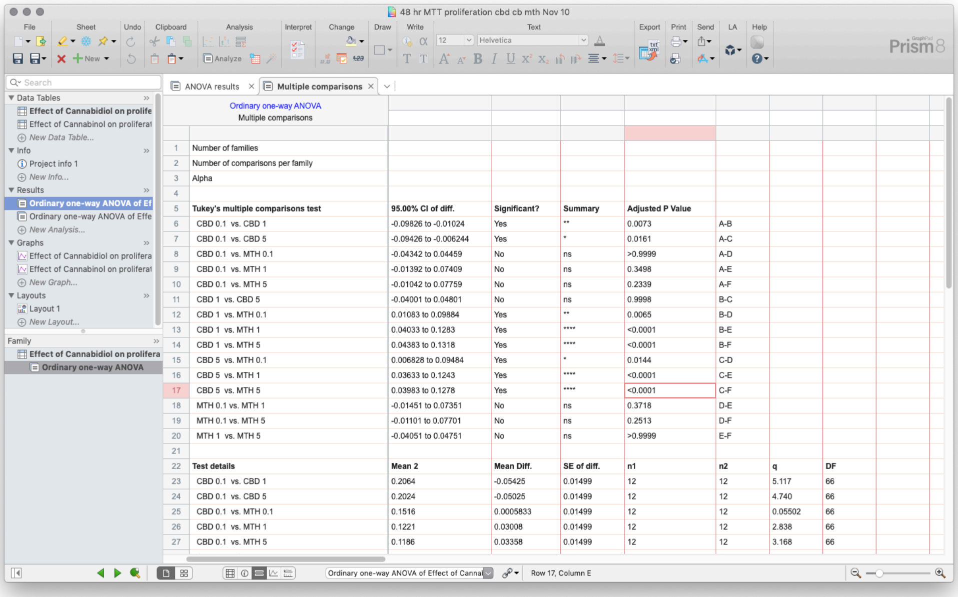

48 hr MTT proliferation cbd cb mth Nov 10
Data Tables
Effect of Cannabidiol on prolifera
Effect of Cannabinol on prolife
Results
Ordinary one-way ANOVA of Effe
Ordinary one-way ANOVA of Ef
Graphs
Effect of Cannabidiol on prolifera
Effect of Cannabinol on prolife
Layouts
Layout 1
Group A
CBN 0.1
Group B
CBN 1
Group C
CBN 5
Group D
MTH 0.1
MTH 1
Mean
SEM
N
0.098083333
0.005814635
12
0.15225
0.00944
12
0.190083333
0.010921523
12
0.115916667
0.008814982
12
0.120666667
Effect of Cannabinol on proliferation (48hr)
Row 1, F: MTH 5



ONE WAY ANOVA Analysis of Proliferation Concentration Results

CBD

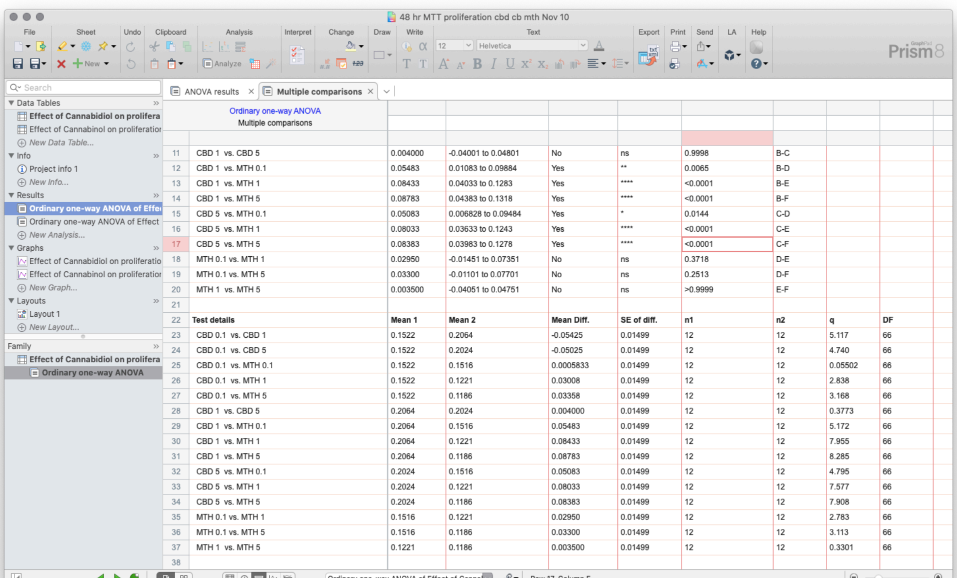




48 hr MTT proliferation cbd cb mth Nov 10

Ordinary one-way ANOVA
Multiple comparisons

1	Number of families							
2	Number of comparisons per family							
3	Alpha							
4								
5	**Tukey's multiple comparisons test**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**			
6	CBD 0.1 vs. CBD 1	-0.09826 to -0.01024	Yes	**	0.0073	A-B		
7	CBD 0.1 vs. CBD 5	-0.09426 to -0.006244	Yes	*	0.0161	A-C		
8	CBD 0.1 vs. MTH 0.1	-0.04342 to 0.04459	No	ns	>0.9999	A-D		
9	CBD 0.1 vs. MTH 1	-0.01392 to 0.07409	No	ns	0.3498	A-E		
10	CBD 0.1 vs. MTH 5	-0.01042 to 0.07759	No	ns	0.2339	A-F		
11	CBD 1 vs. CBD 5	-0.04001 to 0.04801	No	ns	0.9998	B-C		
12	CBD 1 vs. MTH 0.1	0.01083 to 0.09884	Yes	**	0.0065	B-D		
13	CBD 1 vs. MTH 1	0.04033 to 0.1283	Yes	****	<0.0001	B-E		
14	CBD 1 vs. MTH 5	0.04383 to 0.1318	Yes	****	<0.0001	B-F		
15	CBD 5 vs. MTH 0.1	0.006828 to 0.09484	Yes	*	0.0144	C-D		
16	CBD 5 vs. MTH 1	0.03633 to 0.1243	Yes	****	<0.0001	C-E		
17	CBD 5 vs. MTH 5	0.03983 to 0.1278	Yes	****	<0.0001	C-F		
18	MTH 0.1 vs. MTH 1	-0.01451 to 0.07351	No	ns	0.3718	D-E		
19	MTH 0.1 vs. MTH 5	-0.01101 to 0.07701	No	ns	0.2513	D-F		
20	MTH 1 vs. MTH 5	-0.04051 to 0.04751	No	ns	>0.9999	E-F		
21								
22	**Test details**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
23	CBD 0.1 vs. CBD 1	0.2064	-0.05425	0.01499	12	12	5.117	66
24	CBD 0.1 vs. CBD 5	0.2024	-0.05025	0.01499	12	12	4.740	66
25	CBD 0.1 vs. MTH 0.1	0.1516	0.0005833	0.01499	12	12	0.05502	66
26	CBD 0.1 vs. MTH 1	0.1221	0.03008	0.01499	12	12	2.838	66
27	CBD 0.1 vs. MTH 5	0.1186	0.03358	0.01499	12	12	3.168	66

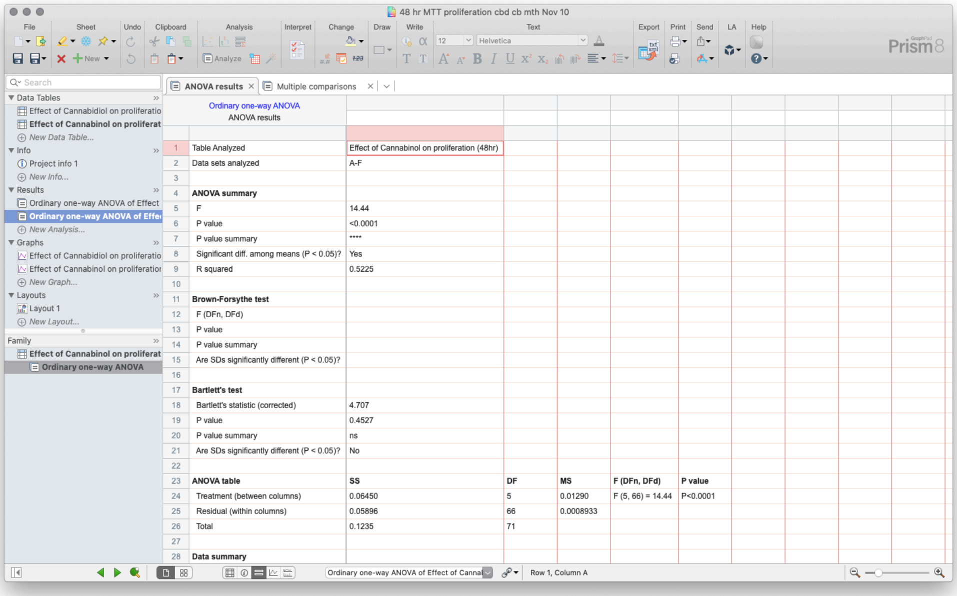


48 hr MTT proliferation cbd cb mth Nov 10

Ordinary one-way ANOVA
Multiple comparisons

11	CBD 1 vs. CBD 5	0.004000	-0.04001 to 0.04801	No	ns	0.9998	B-C		
12	CBD 1 vs. MTH 0.1	0.05483	0.01083 to 0.09884	Yes	**	0.0065	B-D		
13	CBD 1 vs. MTH 1	0.08433	0.04033 to 0.1283	Yes	****	<0.0001	B-E		
14	CBD 1 vs. MTH 5	0.08783	0.04383 to 0.1318	Yes	****	<0.0001	B-F		
15	CBD 5 vs. MTH 0.1	0.05083	0.006828 to 0.09484	Yes	*	0.0144	C-D		
16	CBD 5 vs. MTH 1	0.08033	0.03633 to 0.1243	Yes	****	<0.0001	C-E		
17	CBD 5 vs. MTH 5	0.08383	0.03983 to 0.1278	Yes	****	<0.0001	C-F		
18	MTH 0.1 vs. MTH 1	0.02950	-0.01451 to 0.07351	No	ns	0.3718	D-E		
19	MTH 0.1 vs. MTH 5	0.03300	-0.01101 to 0.07701	No	ns	0.2513	D-F		
20	MTH 1 vs. MTH 5	0.003500	-0.04051 to 0.04751	No	ns	>0.9999	E-F		
21									
22	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
23	CBD 0.1 vs. CBD 1	0.1522	0.2064	-0.05425	0.01499	12	12	5.117	66
24	CBD 0.1 vs. CBD 5	0.1522	0.2024	-0.05025	0.01499	12	12	4.740	66
25	CBD 0.1 vs. MTH 0.1	0.1522	0.1516	0.0005833	0.01499	12	12	0.05502	66
26	CBD 0.1 vs. MTH 1	0.1522	0.1221	0.03008	0.01499	12	12	2.838	66
27	CBD 0.1 vs. MTH 5	0.1522	0.1186	0.03358	0.01499	12	12	3.168	66
28	CBD 1 vs. CBD 5	0.2064	0.2024	0.004000	0.01499	12	12	0.3773	66
29	CBD 1 vs. MTH 0.1	0.2064	0.1516	0.05483	0.01499	12	12	5.172	66
30	CBD 1 vs. MTH 1	0.2064	0.1221	0.08433	0.01499	12	12	7.955	66
31	CBD 1 vs. MTH 5	0.2064	0.1186	0.08783	0.01499	12	12	8.285	66
32	CBD 5 vs. MTH 0.1	0.2024	0.1516	0.05083	0.01499	12	12	4.795	66
33	CBD 5 vs. MTH 1	0.2024	0.1221	0.08033	0.01499	12	12	7.577	66
34	CBD 5 vs. MTH 5	0.2024	0.1186	0.08383	0.01499	12	12	7.908	66
35	MTH 0.1 vs. MTH 1	0.1516	0.1221	0.02950	0.01499	12	12	2.783	66
36	MTH 0.1 vs. MTH 5	0.1516	0.1186	0.03300	0.01499	12	12	3.113	66
37	MTH 1 vs. MTH 5	0.1221	0.1186	0.003500	0.01499	12	12	0.3301	66
38									

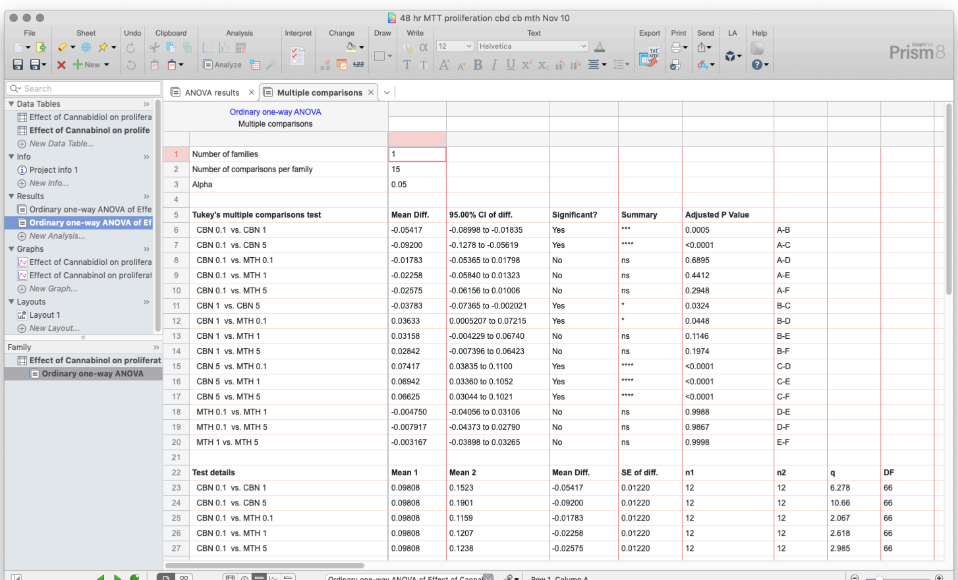
CBN



48 hr MTT proliferation cbd cb mth Nov 10

Ordinary one-way ANOVA
ANOVA results

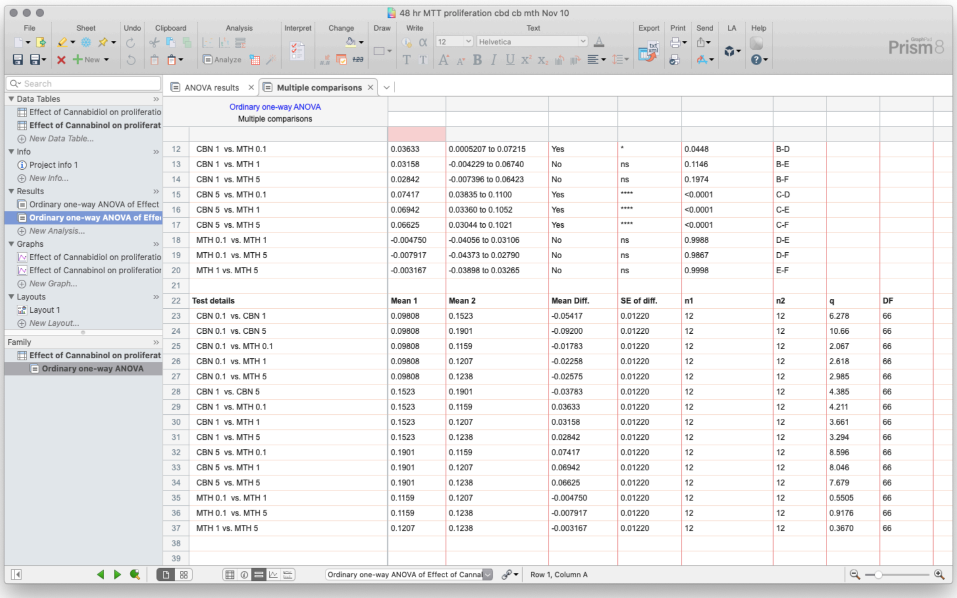
1	Table Analyzed	Effect of Cannabinol on proliferation (48hr)				
2	Data sets analyzed	A-F				
3						
4	**ANOVA summary**					
5	F	14.44				
6	P value	<0.0001				
7	P value summary	****				
8	Significant diff. among means (P < 0.05)?	Yes				
9	R squared	0.5225				
10						
11	**Brown-Forsythe test**					
12	F (DFn, DFd)					
13	P value					
14	P value summary					
15	Are SDs significantly different (P < 0.05)?					
16						
17	**Bartlett's test**					
18	Bartlett's statistic (corrected)	4.707				
19	P value	0.4527				
20	P value summary	ns				
21	Are SDs significantly different (P < 0.05)?	No				
22						
23	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
24	Treatment (between columns)	0.06450	5	0.01290	F (5, 66) = 14.44	P<0.0001
25	Residual (within columns)	0.05896	66	0.0008933		
26	Total	0.1235	71			
27						
28	**Data summary**					



48 hr MTT proliferation cbd cb mth Nov 10

Ordinary one-way ANOVA
Multiple comparisons

1	Number of families	1							
2	Number of comparisons per family	15							
3	Alpha	0.05							
4									
5	**Tukey's multiple comparisons test**	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**			
6	CBN 0.1 vs. CBN 1	-0.05417	-0.08998 to -0.01835	Yes	***	0.0005	A-B		
7	CBN 0.1 vs. CBN 5	-0.09200	-0.1278 to -0.05619	Yes	****	<0.0001	A-C		
8	CBN 0.1 vs. MTH 0.1	-0.01783	-0.05365 to 0.01798	No	ns	0.6895	A-D		
9	CBN 0.1 vs. MTH 1	-0.02258	-0.05840 to 0.01323	No	ns	0.4412	A-E		
10	CBN 0.1 vs. MTH 5	-0.02575	-0.06156 to 0.01006	No	ns	0.2948	A-F		
11	CBN 1 vs. CBN 5	-0.03783	-0.07365 to -0.002021	Yes	*	0.0324	B-C		
12	CBN 1 vs. MTH 0.1	0.03633	0.0005207 to 0.07215	Yes	*	0.0448	B-D		
13	CBN 1 vs. MTH 1	0.03158	-0.004229 to 0.06740	No	ns	0.1146	B-E		
14	CBN 1 vs. MTH 5	0.02842	-0.007396 to 0.06423	No	ns	0.1974	B-F		
15	CBN 5 vs. MTH 0.1	0.07417	0.03835 to 0.1100	Yes	****	<0.0001	C-D		
16	CBN 5 vs. MTH 1	0.06942	0.03360 to 0.1052	Yes	****	<0.0001	C-E		
17	CBN 5 vs. MTH 5	0.06625	0.03044 to 0.1021	Yes	****	<0.0001	C-F		
18	MTH 0.1 vs. MTH 1	-0.004750	-0.04056 to 0.03106	No	ns	0.9988	D-E		
19	MTH 0.1 vs. MTH 5	-0.007917	-0.04373 to 0.02790	No	ns	0.9867	D-F		
20	MTH 1 vs. MTH 5	-0.003167	-0.03898 to 0.03265	No	ns	0.9998	E-F		
21									
22	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
23	CBN 0.1 vs. CBN 1	0.09808	0.1523	-0.05417	0.01220	12	12	6.278	66
24	CBN 0.1 vs. CBN 5	0.09808	0.1901	-0.09200	0.01220	12	12	10.66	66
25	CBN 0.1 vs. MTH 0.1	0.09808	0.1159	-0.01783	0.01220	12	12	2.067	66
26	CBN 0.1 vs. MTH 1	0.09808	0.1207	-0.02258	0.01220	12	12	2.618	66
27	CBN 0.1 vs. MTH 5	0.09808	0.1238	-0.02575	0.01220	12	12	2.985	66



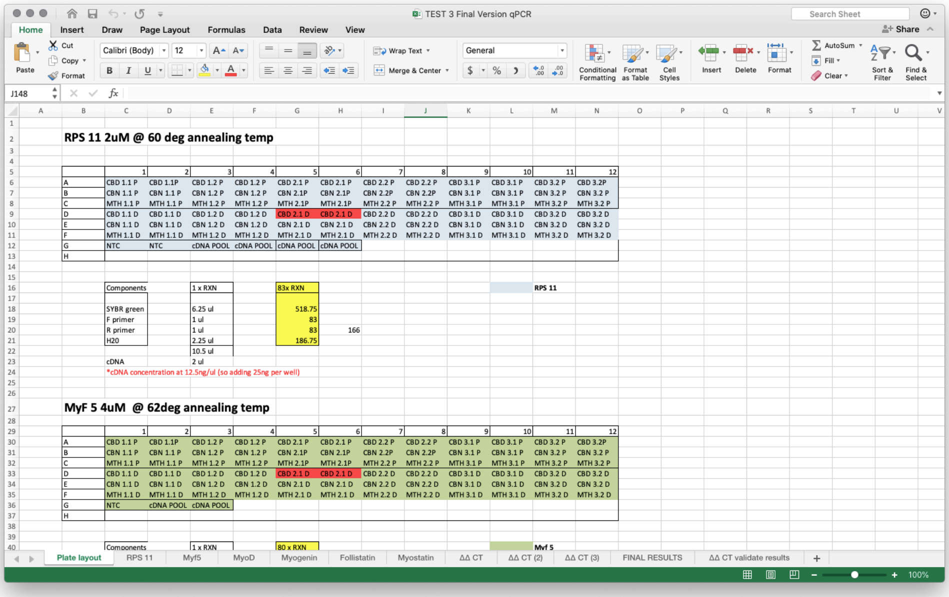
48 hr MTT proliferation cbd cb mth Nov 10

Ordinary one-way ANOVA
Multiple comparisons

12	CBN 1 vs. MTH 0.1	0.03633	0.0005207 to 0.07215	Yes	*	0.0448	B-D		
13	CBN 1 vs. MTH 1	0.03158	-0.004229 to 0.06740	No	ns	0.1146	B-E		
14	CBN 1 vs. MTH 5	0.02842	-0.007396 to 0.06423	No	ns	0.1974	B-F		
15	CBN 5 vs. MTH 0.1	0.07417	0.03835 to 0.1100	Yes	****	<0.0001	C-D		
16	CBN 5 vs. MTH 1	0.06942	0.03360 to 0.1052	Yes	****	<0.0001	C-E		
17	CBN 5 vs. MTH 5	0.06625	0.03044 to 0.1021	Yes	****	<0.0001	C-F		
18	MTH 0.1 vs. MTH 1	-0.004750	-0.04056 to 0.03106	No	ns	0.9988	D-E		
19	MTH 0.1 vs. MTH 5	-0.007917	-0.04373 to 0.02790	No	ns	0.9867	D-F		
20	MTH 1 vs. MTH 5	-0.003167	-0.03898 to 0.03265	No	ns	0.9998	E-F		
21									
22	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
23	CBN 0.1 vs. CBN 1	0.09808	0.1523	-0.05417	0.01220	12	12	6.278	66
24	CBN 0.1 vs. CBN 5	0.09808	0.1901	-0.09200	0.01220	12	12	10.66	66
25	CBN 0.1 vs. MTH 0.1	0.09808	0.1159	-0.01783	0.01220	12	12	2.067	66
26	CBN 0.1 vs. MTH 1	0.09808	0.1207	-0.02258	0.01220	12	12	2.618	66
27	CBN 0.1 vs. MTH 5	0.09808	0.1238	-0.02575	0.01220	12	12	2.985	66
28	CBN 1 vs. CBN 5	0.1523	0.1901	-0.03783	0.01220	12	12	4.385	66
29	CBN 1 vs. MTH 0.1	0.1523	0.1159	0.03633	0.01220	12	12	4.211	66
30	CBN 1 vs. MTH 1	0.1523	0.1207	0.03158	0.01220	12	12	3.661	66
31	CBN 1 vs. MTH 5	0.1523	0.1238	0.02842	0.01220	12	12	3.294	66
32	CBN 5 vs. MTH 0.1	0.1901	0.1159	0.07417	0.01220	12	12	8.596	66
33	CBN 5 vs. MTH 1	0.1901	0.1207	0.06942	0.01220	12	12	8.046	66
34	CBN 5 vs. MTH 5	0.1901	0.1238	0.06625	0.01220	12	12	7.679	66
35	MTH 0.1 vs. MTH 1	0.1159	0.1207	-0.004750	0.01220	12	12	0.5505	66
36	MTH 0.1 vs. MTH 5	0.1159	0.1238	-0.007917	0.01220	12	12	0.9176	66
37	MTH 1 vs. MTH 5	0.1207	0.1238	-0.003167	0.01220	12	12	0.3670	66

PLATE LAYOUT Real Time-PCR (5uM)

TRIAL 3



RPS 11 2uM @ 60 deg annealing temp

	1	2	3	4	5	6	7	8	9	10	11	12
A	CBD 1.1 P	CBD 1.1P	CBD 1.2 P	CBD 1.2 P	CBD 2.1 P	CBD 2.1 P	CBD 2.2 P	CBD 2.2 P	CBD 3.1 P	CBD 3.1 P	CBD 3.2 P	CBD 3.2P
B	CBN 1.1 P	CBN 1.1 P	CBN 1.2 P	CBN 1.2 P	CBN 2.1P	CBN 2.1P	CBN 2.2P	CBN 2.2P	CBN 3.1 P	CBN 3.1 P	CBN 3.2 P	CBN 3.2 P
C	MTH 1.1 P	MTH 1.1 P	MTH 1.2 P	MTH 1.2 P	MTH 2.1P	MTH 2.1P	MTH 2.2 P	MTH 2.2 P	MTH 3.1 P	MTH 3.1 P	MTH 3.2 P	MTH 3.2 P
D	CBD 1.1 D	CBD 1.1 D	CBD 1.2 D	CBD 1.2 D	CBD 2.1 D	CBD 2.1 D	CBD 2.2 D	CBD 2.2 D	CBD 3.1 D	CBD 3.1 D	CBD 3.2 D	CBD 3.2 D
E	CBN 1.1 D	CBN 1.1 D	CBN 1.2 D	CBN 1.2 D	CBN 2.1 D	CBN 2.1 D	CBN 2.2 D	CBN 2.2 D	CBN 3.1 D	CBN 3.1 D	CBN 3.2 D	CBN 3.2 D
F	MTH 1.1 D	MTH 1.1 D	MTH 1.2 D	MTH 1.2 D	MTH 2.1 D	MTH 2.1 D	MTH 2.2 D	MTH 2.2 D	MTH 3.1 D	MTH 3.1 D	MTH 3.2 D	MTH 3.2 D
G	NTC	NTC	cDNA POOL	cDNA POOL	cDNA POOL	cDNA POOL						
H												

Components	1 x RXN	83x RXN	
SYBR green	6.25 ul	518.75	
F primer	1 ul	83	
R primer	1 ul	83	166
H20	2.25 ul	186.75	
	10.5 ul		
cDNA	2 ul		

*cDNA concentration at 12.5ng/ul (so adding 25ng per well)

RPS 11

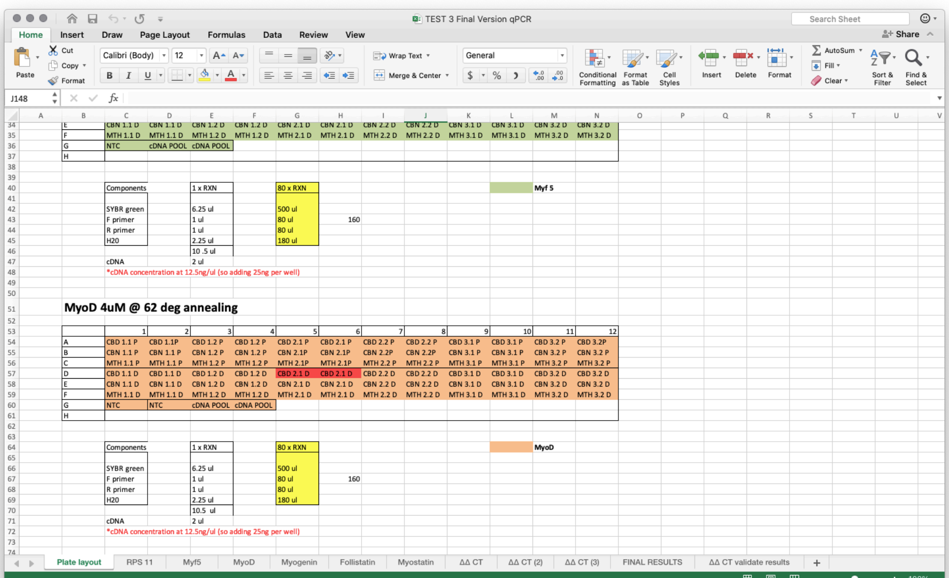
MyF 5 4uM @ 62deg annealing temp

	1	2	3	4	5	6	7	8	9	10	11	12
A	CBD 1.1 P	CBD 1.1P	CBD 1.2 P	CBD 1.2 P	CBD 2.1 P	CBD 2.1 P	CBD 2.2 P	CBD 2.2 P	CBD 3.1 P	CBD 3.1 P	CBD 3.2 P	CBD 3.2P
B	CBN 1.1 P	CBN 1.1 P	CBN 1.2 P	CBN 1.2 P	CBN 2.1P	CBN 2.1P	CBN 2.2P	CBN 2.2P	CBN 3.1 P	CBN 3.1 P	CBN 3.2 P	CBN 3.2 P
C	MTH 1.1 P	MTH 1.1 P	MTH 1.2 P	MTH 1.2 P	MTH 2.1P	MTH 2.1P	MTH 2.2 P	MTH 2.2 P	MTH 3.1 P	MTH 3.1 P	MTH 3.2 P	MTH 3.2 P
D	CBD 1.1 D	CBD 1.1 D	CBD 1.2 D	CBD 1.2 D	CBD 2.1 D	CBD 2.1 D	CBD 2.2 D	CBD 2.2 D	CBD 3.1 D	CBD 3.1 D	CBD 3.2 D	CBD 3.2 D
E	CBN 1.1 D	CBN 1.1 D	CBN 1.2 D	CBN 1.2 D	CBN 2.1 D	CBN 2.1 D	CBN 2.2 D	CBN 2.2 D	CBN 3.1 D	CBN 3.1 D	CBN 3.2 D	CBN 3.2 D
F	MTH 1.1 D	MTH 1.1 D	MTH 1.2 D	MTH 1.2 D	MTH 2.1 D	MTH 2.1 D	MTH 2.2 D	MTH 2.2 D	MTH 3.1 D	MTH 3.1 D	MTH 3.2 D	MTH 3.2 D
G	NTC	cDNA POOL	cDNA POOL									
H												

Components	1 x RXN	80 x RXN

Myf 5

Plate layout | RPS 11 | Myf5 | MyoD | Myogenin | Follistatin | Myostatin | ΔΔ CT | ΔΔ CT (2) | ΔΔ CT (3) | FINAL RESULTS | ΔΔ CT validate results



E	CBN 1.1 D	CBN 1.1 D	CBN 1.2 D	CBN 1.2 D	CBN 2.1 D	CBN 2.1 D	CBN 2.2 D	CBN 2.2 D	CBN 3.1 D	CBN 3.1 D	CBN 3.2 D	CBN 3.2 D
F	MTH 1.1 D	MTH 1.1 D	MTH 1.2 D	MTH 1.2 D	MTH 2.1 D	MTH 2.1 D	MTH 2.2 D	MTH 2.2 D	MTH 3.1 D	MTH 3.1 D	MTH 3.2 D	MTH 3.2 D
G	NTC	cDNA POOL	cDNA POOL									
H												

Components	1 x RXN	80 x RXN	
SYBR green	6.25 ul	500 ul	
F primer	1 ul	80 ul	160
R primer	1 ul	80 ul	
H20	2.25 ul	180 ul	
	10 .5 ul		
cDNA	2 ul		

*cDNA concentration at 12.5ng/ul (so adding 25ng per well)

Myf 5

MyoD 4uM @ 62 deg annealing

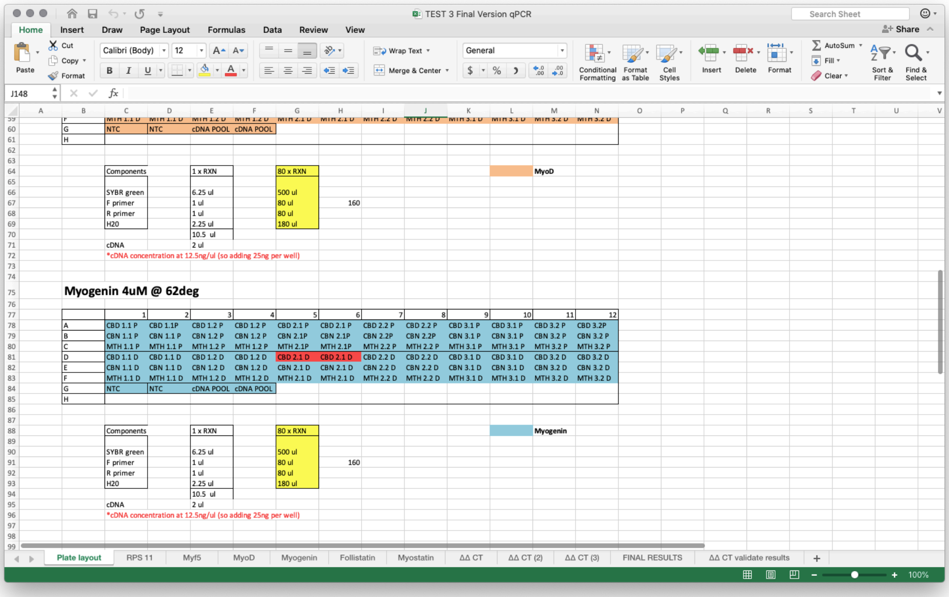
	1	2	3	4	5	6	7	8	9	10	11	12
A	CBD 1.1 P	CBD 1.1P	CBD 1.2 P	CBD 1.2 P	CBD 2.1 P	CBD 2.1 P	CBD 2.2 P	CBD 2.2 P	CBD 3.1 P	CBD 3.1 P	CBD 3.2 P	CBD 3.2P
B	CBN 1.1 P	CBN 1.1 P	CBN 1.2 P	CBN 1.2 P	CBN 2.1P	CBN 2.1P	CBN 2.2P	CBN 2.2P	CBN 3.1 P	CBN 3.1 P	CBN 3.2 P	CBN 3.2 P
C	MTH 1.1 P	MTH 1.1 P	MTH 1.2 P	MTH 1.2 P	MTH 2.1P	MTH 2.1P	MTH 2.2 P	MTH 2.2 P	MTH 3.1 P	MTH 3.1 P	MTH 3.2 P	MTH 3.2 P
D	CBD 1.1 D	CBD 1.1 D	CBD 1.2 D	CBD 1.2 D	CBD 2.1 D	CBD 2.1 D	CBD 2.2 D	CBD 2.2 D	CBD 3.1 D	CBD 3.1 D	CBD 3.2 D	CBD 3.2 D
E	CBN 1.1 D	CBN 1.1 D	CBN 1.2 D	CBN 1.2 D	CBN 2.1 D	CBN 2.1 D	CBN 2.2 D	CBN 2.2 D	CBN 3.1 D	CBN 3.1 D	CBN 3.2 D	CBN 3.2 D
F	MTH 1.1 D	MTH 1.1 D	MTH 1.2 D	MTH 1.2 D	MTH 2.1 D	MTH 2.1 D	MTH 2.2 D	MTH 2.2 D	MTH 3.1 D	MTH 3.1 D	MTH 3.2 D	MTH 3.2 D
G	NTC	NTC	cDNA POOL	cDNA POOL								
H												

Components	1 x RXN	80 x RXN	
SYBR green	6.25 ul	500 ul	
F primer	1 ul	80 ul	160
R primer	1 ul	80 ul	
H20	2.25 ul	180 ul	
	10.5 ul		
cDNA	2 ul		

*cDNA concentration at 12.5ng/ul (so adding 25ng per well)

MyoD

Plate layout | RPS 11 | Myf5 | MyoD | Myogenin | Follistatin | Myostatin | ΔΔ CT | ΔΔ CT (2) | ΔΔ CT (3) | FINAL RESULTS | ΔΔ CT validate results



TEST 3 Final Version qPCR

G	NTC	NTC	cDNA POOL	cDNA POOL								
H												

Components	1 x RXN	80 x RXN	
SYBR green	6.25 ul	500 ul	
F primer	1 ul	80 ul	160
R primer	1 ul	80 ul	
H20	2.25 ul	180 ul	
	10.5 ul		
cDNA	2 ul		

*cDNA concentration at 12.5ng/ul (so adding 25ng per well)

MyoD

Myogenin 4uM @ 62deg

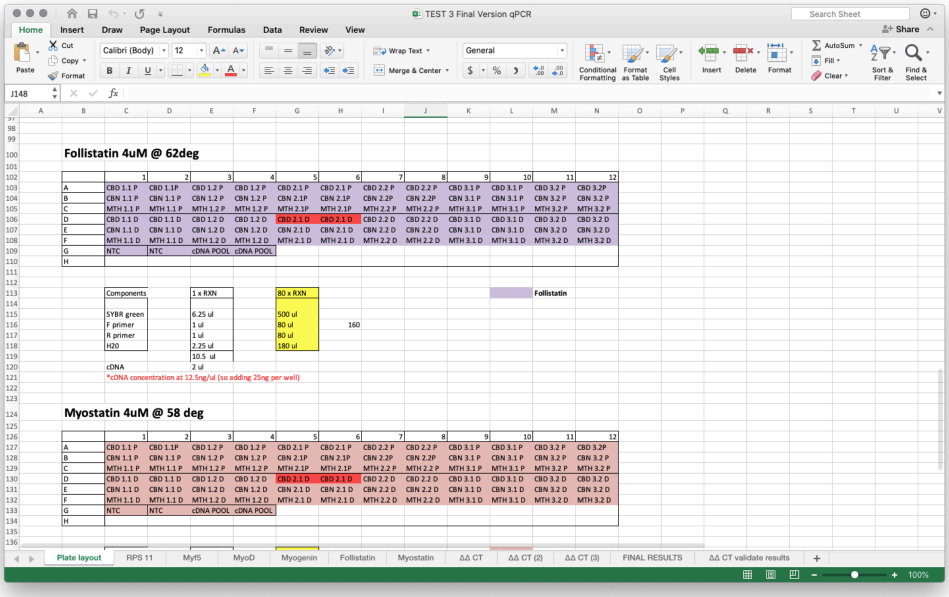
	1	2	3	4	5	6	7	8	9	10	11	12
A	CBD 1.1 P	CBD 1.1P	CBD 1.2 P	CBD 1.2 P	CBD 2.1 P	CBD 2.1 P	CBD 2.2 P	CBD 2.2 P	CBD 3.1 P	CBD 3.1 P	CBD 3.2 P	CBD 3.2P
B	CBN 1.1 P	CBN 1.1 P	CBN 1.2 P	CBN 1.2 P	CBN 2.1P	CBN 2.1P	CBN 2.2P	CBN 2.2P	CBN 3.1 P	CBN 3.1 P	CBN 3.2 P	CBN 3.2 P
C	MTH 1.1 P	MTH 1.1 P	MTH 1.2 P	MTH 1.2 P	MTH 2.1P	MTH 2.1P	MTH 2.2 P	MTH 2.2 P	MTH 3.1 P	MTH 3.1 P	MTH 3.2 P	MTH 3.2 P
D	CBD 1.1 D	CBD 1.1 D	CBD 1.2 D	CBD 1.2 D	CBD 2.1 D	CBD 2.1 D	CBD 2.2 D	CBD 2.2 D	CBD 3.1 D	CBD 3.1 D	CBD 3.2 D	CBD 3.2 D
E	CBN 1.1 D	CBN 1.1 D	CBN 1.2 D	CBN 1.2 D	CBN 2.1 D	CBN 2.1 D	CBN 2.2 D	CBN 2.2 D	CBN 3.1 D	CBN 3.1 D	CBN 3.2 D	CBN 3.2 D
F	MTH 1.1 D	MTH 1.1 D	MTH 1.2 D	MTH 1.2 D	MTH 2.1 D	MTH 2.1 D	MTH 2.2 D	MTH 2.2 D	MTH 3.1 D	MTH 3.1 D	MTH 3.2 D	MTH 3.2 D
G	NTC	NTC	cDNA POOL	cDNA POOL								
H												

Components	1 x RXN	80 x RXN	
SYBR green	6.25 ul	500 ul	
F primer	1 ul	80 ul	160
R primer	1 ul	80 ul	
H20	2.25 ul	180 ul	
	10.5 ul		
cDNA	2 ul		

*cDNA concentration at 12.5ng/ul (so adding 25ng per well)

Myogenin

Plate layout | RPS 11 | Myf5 | MyoD | Myogenin | Follistatin | Myostatin | ΔΔ CT | ΔΔ CT (2) | ΔΔ CT (3) | FINAL RESULTS | ΔΔ CT validate results



Follistatin 4uM @ 62deg

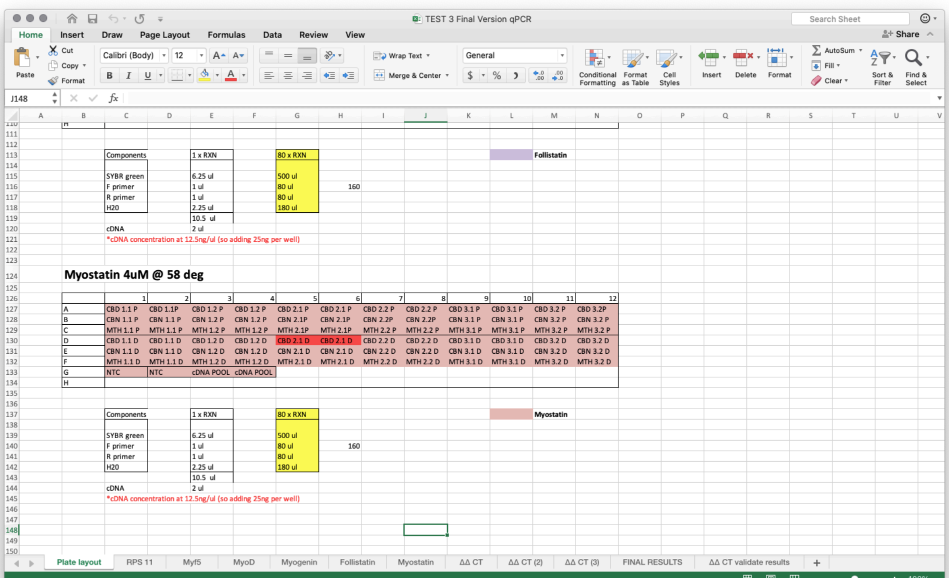
	1	2	3	4	5	6	7	8	9	10	11	12
A	CBD 1.1 P	CBD 1.1P	CBD 1.2 P	CBD 1.2 P	CBD 2.1 P	CBD 2.1 P	CBD 2.2 P	CBD 2.2 P	CBD 3.1 P	CBD 3.1 P	CBD 3.2 P	CBD 3.2P
B	CBN 1.1 P	CBN 1.1 P	CBN 1.2 P	CBN 1.2 P	CBN 2.1P	CBN 2.1P	CBN 2.2P	CBN 2.2P	CBN 3.1 P	CBN 3.1 P	CBN 3.2 P	CBN 3.2 P
C	MTH 1.1 P	MTH 1.1 P	MTH 1.2 P	MTH 1.2 P	MTH 2.1P	MTH 2.1P	MTH 2.2 P	MTH 2.2 P	MTH 3.1 P	MTH 3.1 P	MTH 3.2 P	MTH 3.2 P
D	CBD 1.1 D	CBD 1.1 D	CBD 1.2 D	CBD 1.2 D	CBD 2.1 D	CBD 2.1 D	CBD 2.2 D	CBD 2.2 D	CBD 3.1 D	CBD 3.1 D	CBD 3.2 D	CBD 3.2 D
E	CBN 1.1 D	CBN 1.1 D	CBN 1.2 D	CBN 1.2 D	CBN 2.1 D	CBN 2.1 D	CBN 2.2 D	CBN 2.2 D	CBN 3.1 D	CBN 3.1 D	CBN 3.2 D	CBN 3.2 D
F	MTH 1.1 D	MTH 1.1 D	MTH 1.2 D	MTH 1.2 D	MTH 2.1 D	MTH 2.1 D	MTH 2.2 D	MTH 2.2 D	MTH 3.1 D	MTH 3.1 D	MTH 3.2 D	MTH 3.2 D
G	NTC	NTC	cDNA POOL	cDNA POOL								
H												

Components	1 x RXN	80 x RXN		
				Follistatin
SYBR green	6.25 ul	500 ul		
F primer	1 ul	80 ul	160	
R primer	1 ul	80 ul		
H20	2.25 ul	180 ul		
	10.5 ul			
cDNA	2 ul			

*cDNA concentration at 12.5ng/ul (so adding 25ng per well)

Myostatin 4uM @ 58 deg

	1	2	3	4	5	6	7	8	9	10	11	12
A	CBD 1.1 P	CBD 1.1P	CBD 1.2 P	CBD 1.2 P	CBD 2.1 P	CBD 2.1 P	CBD 2.2 P	CBD 2.2 P	CBD 3.1 P	CBD 3.1 P	CBD 3.2 P	CBD 3.2P
B	CBN 1.1 P	CBN 1.1 P	CBN 1.2 P	CBN 1.2 P	CBN 2.1P	CBN 2.1P	CBN 2.2P	CBN 2.2P	CBN 3.1 P	CBN 3.1 P	CBN 3.2 P	CBN 3.2 P
C	MTH 1.1 P	MTH 1.1 P	MTH 1.2 P	MTH 1.2 P	MTH 2.1P	MTH 2.1P	MTH 2.2 P	MTH 2.2 P	MTH 3.1 P	MTH 3.1 P	MTH 3.2 P	MTH 3.2 P
D	CBD 1.1 D	CBD 1.1 D	CBD 1.2 D	CBD 1.2 D	CBD 2.1 D	CBD 2.1 D	CBD 2.2 D	CBD 2.2 D	CBD 3.1 D	CBD 3.1 D	CBD 3.2 D	CBD 3.2 D
E	CBN 1.1 D	CBN 1.1 D	CBN 1.2 D	CBN 1.2 D	CBN 2.1 D	CBN 2.1 D	CBN 2.2 D	CBN 2.2 D	CBN 3.1 D	CBN 3.1 D	CBN 3.2 D	CBN 3.2 D
F	MTH 1.1 D	MTH 1.1 D	MTH 1.2 D	MTH 1.2 D	MTH 2.1 D	MTH 2.1 D	MTH 2.2 D	MTH 2.2 D	MTH 3.1 D	MTH 3.1 D	MTH 3.2 D	MTH 3.2 D
G	NTC	NTC	cDNA POOL	cDNA POOL								
H												



Components	1 x RXN	80 x RXN		
				Follistatin
SYBR green	6.25 ul	500 ul		
F primer	1 ul	80 ul	160	
R primer	1 ul	80 ul		
H20	2.25 ul	180 ul		
	10.5 ul			
cDNA	2 ul			

*cDNA concentration at 12.5ng/ul (so adding 25ng per well)

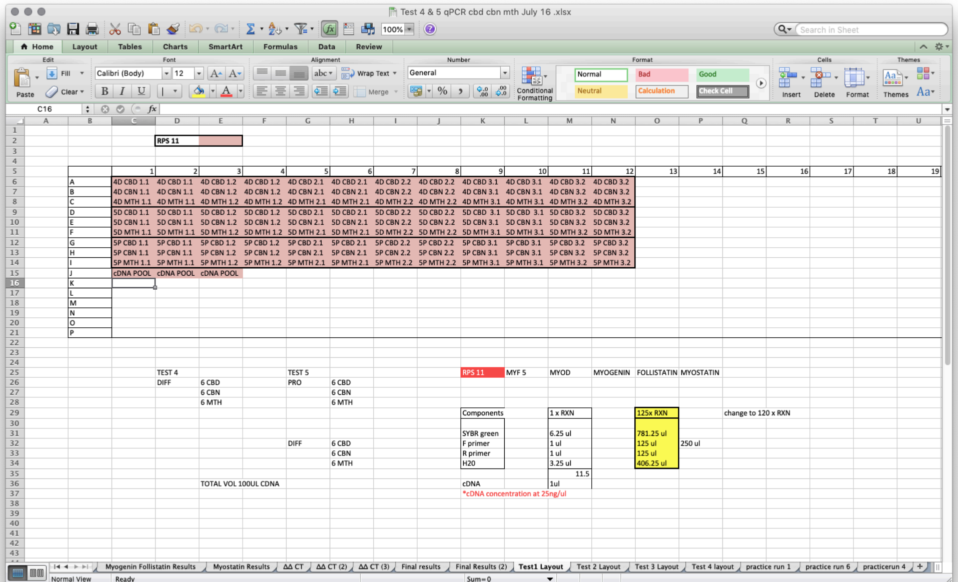
Myostatin 4uM @ 58 deg

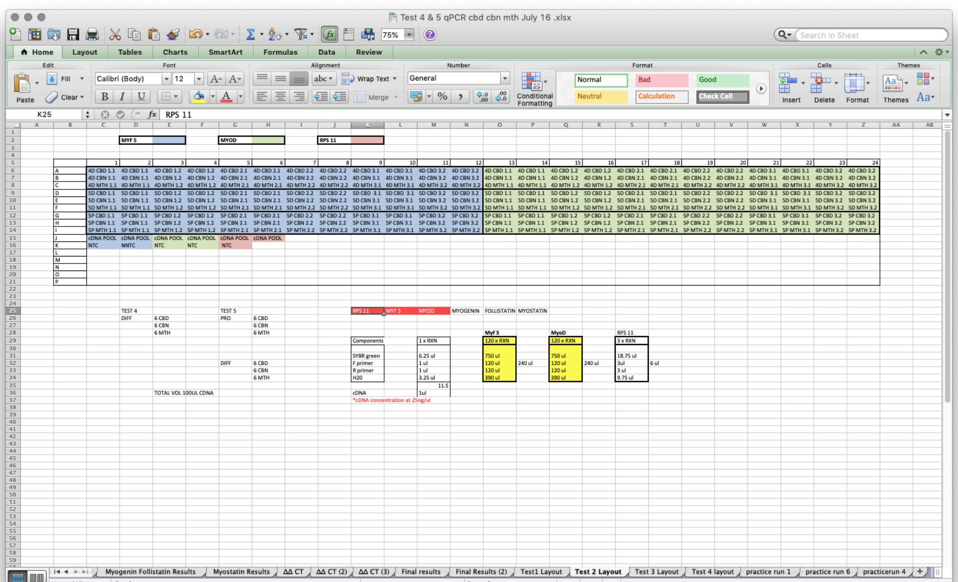
	1	2	3	4	5	6	7	8	9	10	11	12
A	CBD 1.1 P	CBD 1.1P	CBD 1.2 P	CBD 1.2 P	CBD 2.1 P	CBD 2.1 P	CBD 2.2 P	CBD 2.2 P	CBD 3.1 P	CBD 3.1 P	CBD 3.2 P	CBD 3.2P
B	CBN 1.1 P	CBN 1.1 P	CBN 1.2 P	CBN 1.2 P	CBN 2.1P	CBN 2.1P	CBN 2.2P	CBN 2.2P	CBN 3.1 P	CBN 3.1 P	CBN 3.2 P	CBN 3.2 P
C	MTH 1.1 P	MTH 1.1 P	MTH 1.2 P	MTH 1.2 P	MTH 2.1P	MTH 2.1P	MTH 2.2 P	MTH 2.2 P	MTH 3.1 P	MTH 3.1 P	MTH 3.2 P	MTH 3.2 P
D	CBD 1.1 D	CBD 1.1 D	CBD 1.2 D	CBD 1.2 D	CBD 2.1 D	CBD 2.1 D	CBD 2.2 D	CBD 2.2 D	CBD 3.1 D	CBD 3.1 D	CBD 3.2 D	CBD 3.2 D
E	CBN 1.1 D	CBN 1.1 D	CBN 1.2 D	CBN 1.2 D	CBN 2.1 D	CBN 2.1 D	CBN 2.2 D	CBN 2.2 D	CBN 3.1 D	CBN 3.1 D	CBN 3.2 D	CBN 3.2 D
F	MTH 1.1 D	MTH 1.1 D	MTH 1.2 D	MTH 1.2 D	MTH 2.1 D	MTH 2.1 D	MTH 2.2 D	MTH 2.2 D	MTH 3.1 D	MTH 3.1 D	MTH 3.2 D	MTH 3.2 D
G	NTC	NTC	cDNA POOL	cDNA POOL								
H												

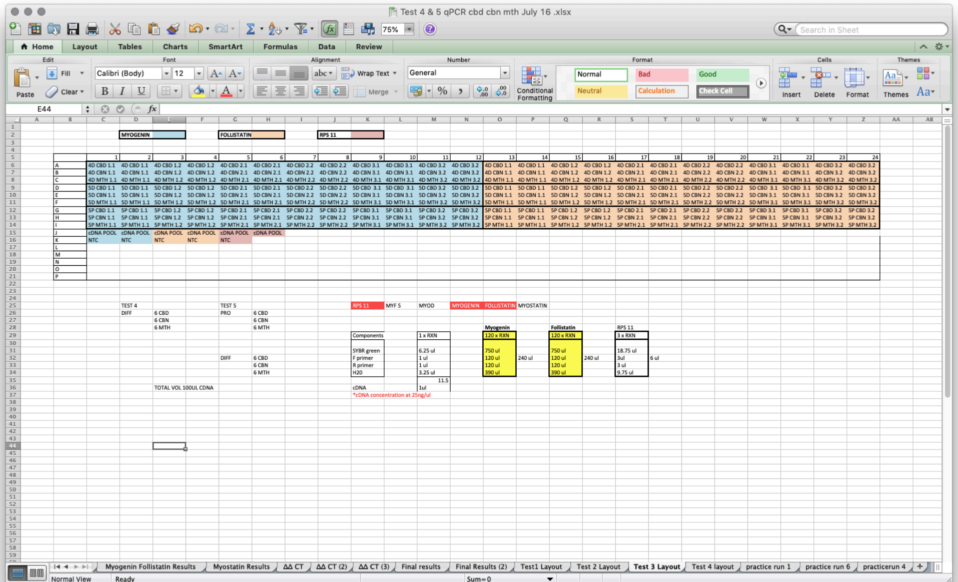
Components	1 x RXN	80 x RXN		
				Myostatin
SYBR green	6.25 ul	500 ul		
F primer	1 ul	80 ul	160	
R primer	1 ul	80 ul		
H20	2.25 ul	180 ul		
	10.5 ul			
cDNA	2 ul			

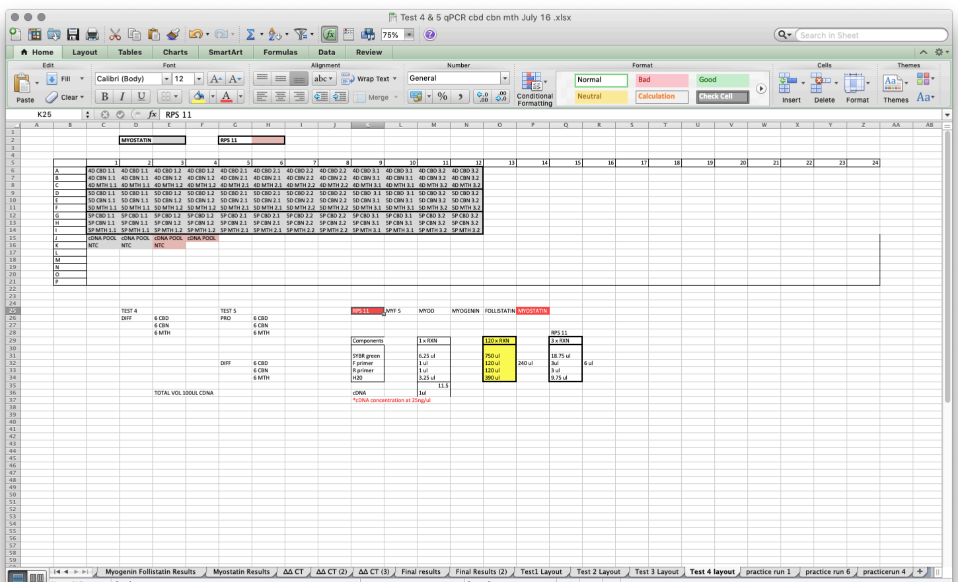
*cDNA concentration at 12.5ng/ul (so adding 25ng per well)

TRIAL 4 & 5

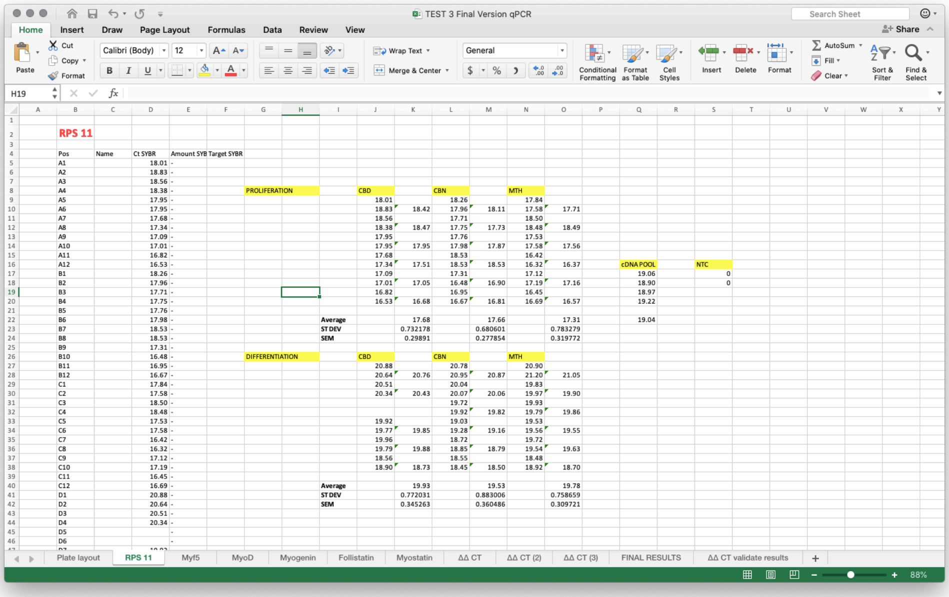

Test 4 & 5 qPCR cbd cbn mth July 16 .xlsx
RPS 11
TEST 4
TEST 5
Myogenin Follistatin Results
Myostatin Results
Test1 Layout
Test 2 Layout
Test 3 Layout
Test 4 layout

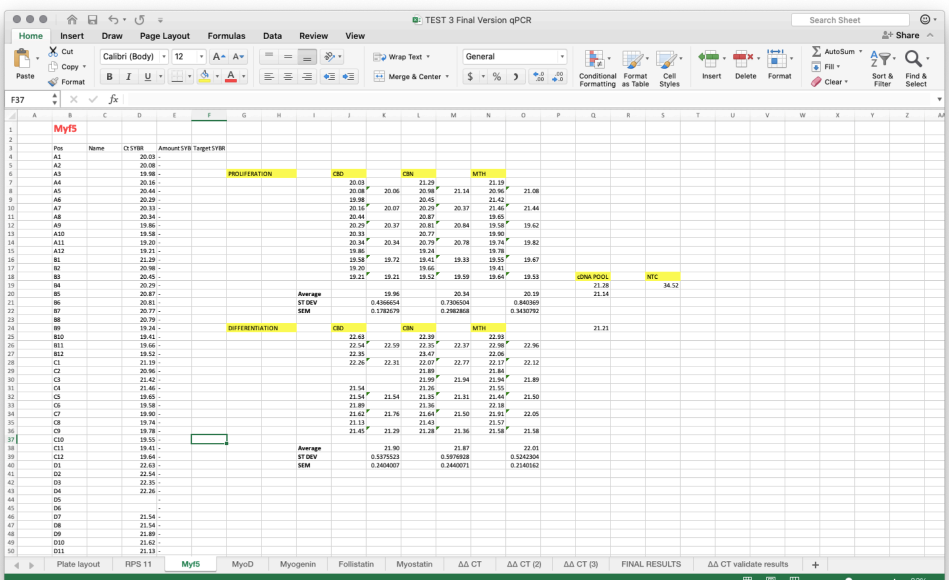

Test 4 & 5 qPCR cbd cbn mth July 16 .xlsx
MYF 5
MYOD
RPS 11
Test 2 Layout

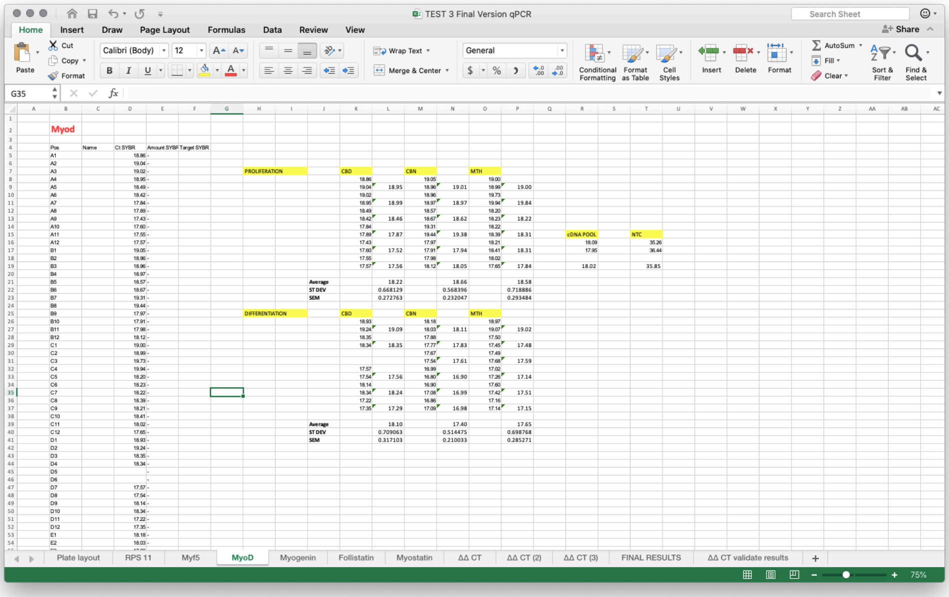


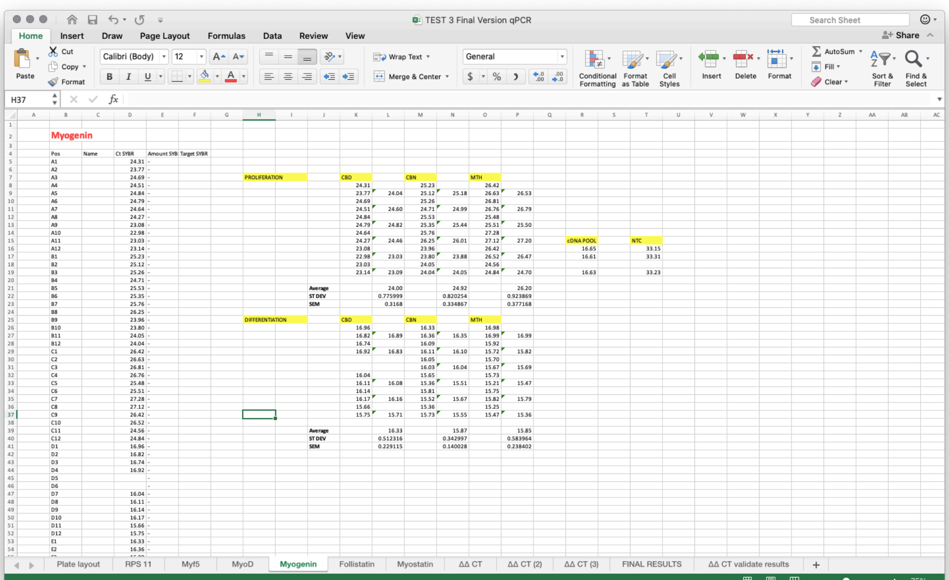


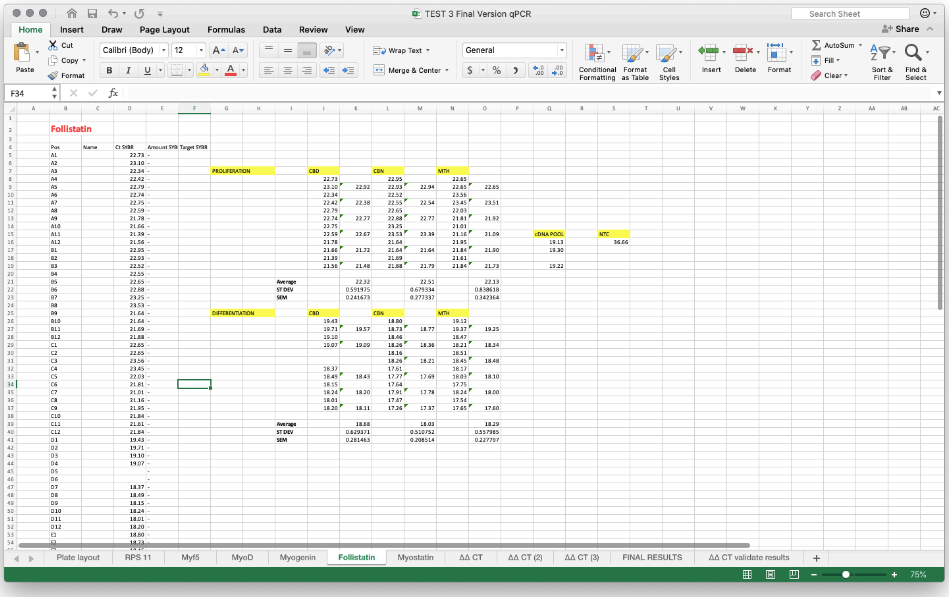
RAW DATA Real Time-PCR Results (5uM) TRIAL 3

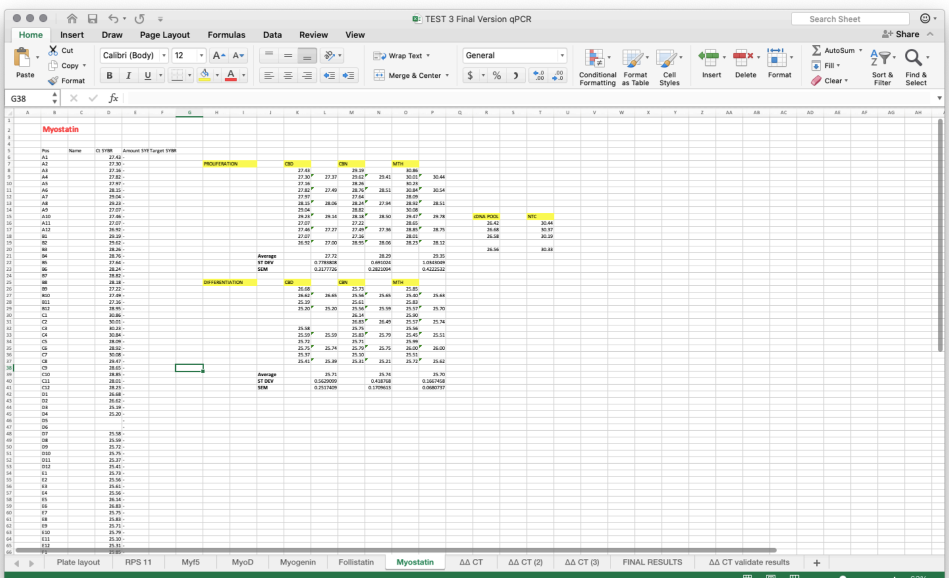

TEST 3 Final Version qPCR
RPS 11
PROLIFERATION
DIFFERENTIATION
CBD
CBN
MTH
cDNA POOL
NTC
Average
ST DEV
SEM
Plate layout
RPS 11
Myf5
MyoD
Myogenin
Follistatin
Myostatin
ΔΔ CT
ΔΔ CT (2)
ΔΔ CT (3)
FINAL RESULTS
ΔΔ CT validate results


TEST 3 Final Version qPCR
Myf5
PROLIFERATION
DIFFERENTIATION
CBD
CBN
MTH
cDNA POOL
NTC
Average
ST DEV
SEM
Plate layout
RPS 11
Myf5
MyoD
Myogenin
Follistatin
Myostatin
ΔΔ CT
ΔΔ CT (2)
ΔΔ CT (3)
FINAL RESULTS
ΔΔ CT validate results

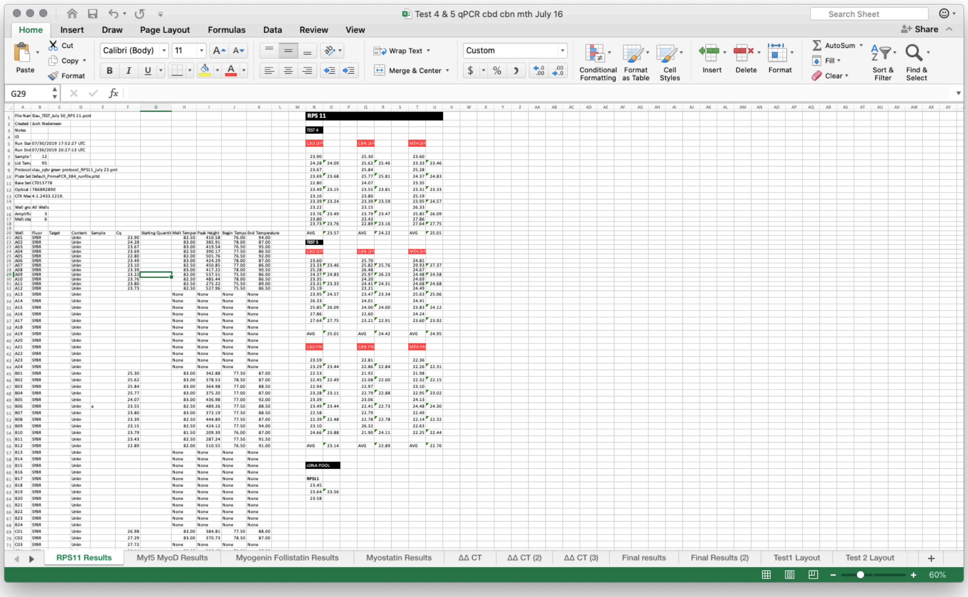

TEST 3 Final Version qPCR
Myod
PROLIFERATION
DIFFERENTIATION
CBD
CBN
MTH
Average
ST DEV
SEM
cDNA POOL
NTC
Plate layout
RPS 11
Myf5
MyoD
Myogenin
Follistatin
Myostatin
FINAL RESULTS

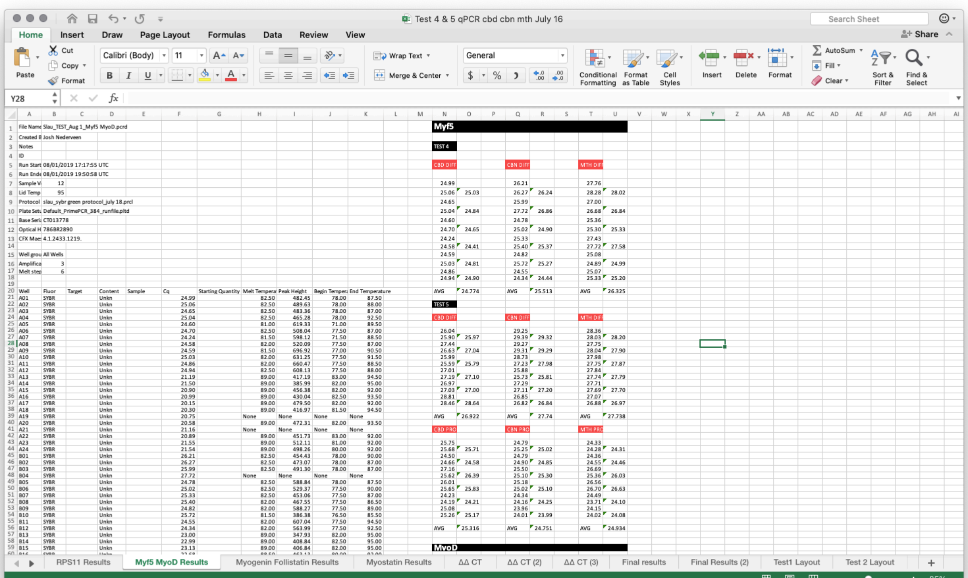

TEST 3 Final Version qPCR
Myogenin
PROLIFERATION
DIFFERENTIATION
CBD
CBN
MTH
Average
ST DEV
SEM
cDNA POOL
NTC
Plate layout
RPS 11
Myf5
MyoD
Myogenin
Follistatin
Myostatin
FINAL RESULTS

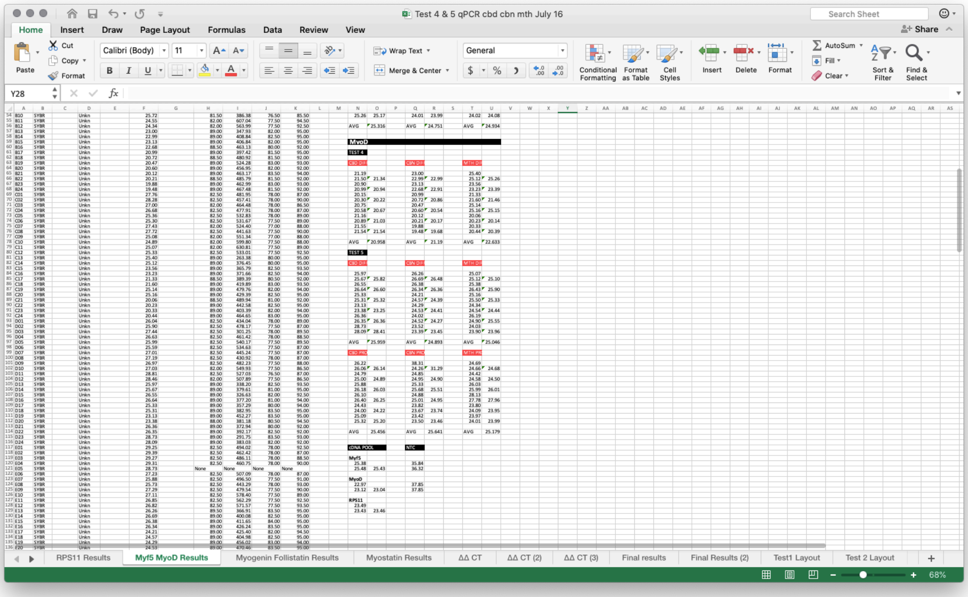


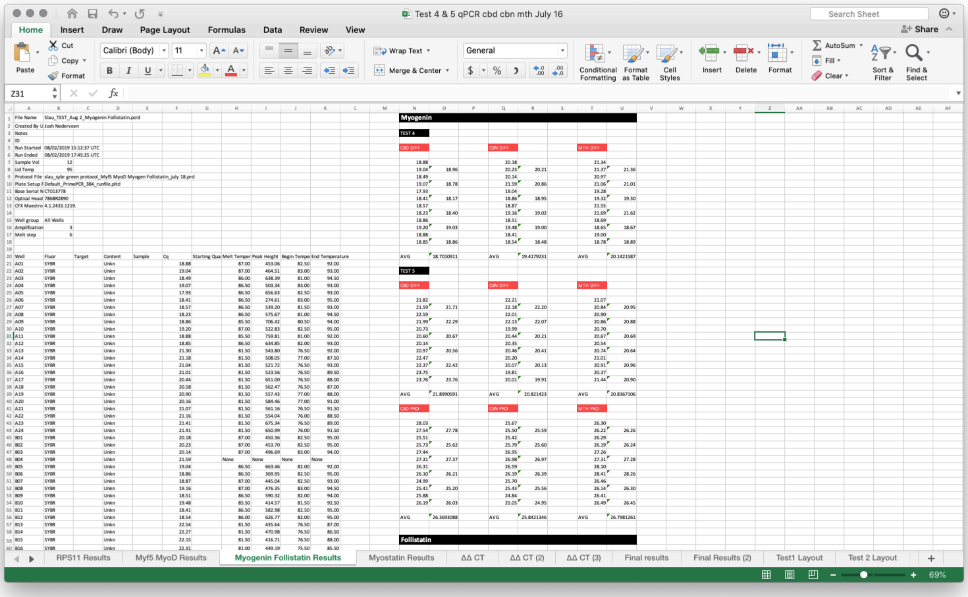


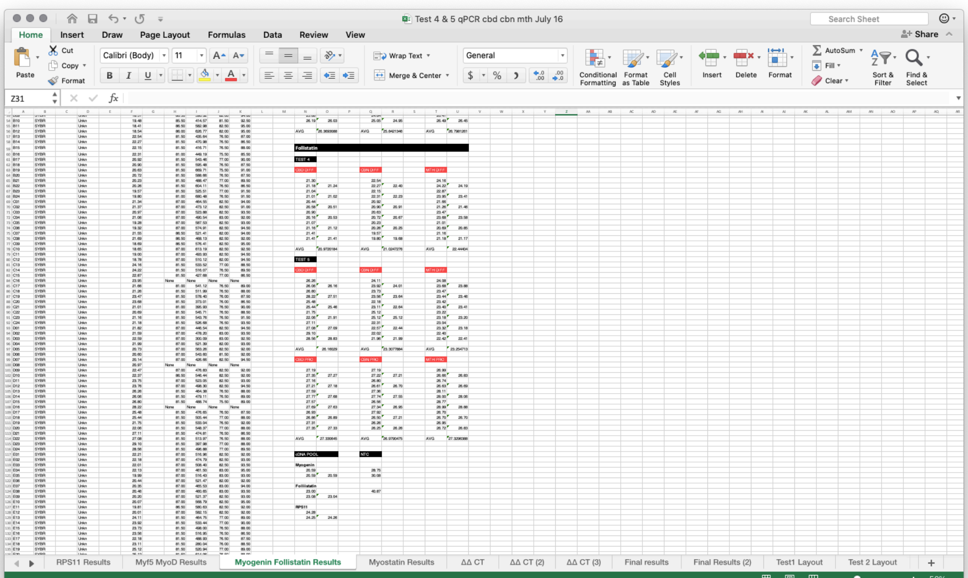
RAW DATA Real Time-PCR Results (5uM) TRIAL 4 & 5

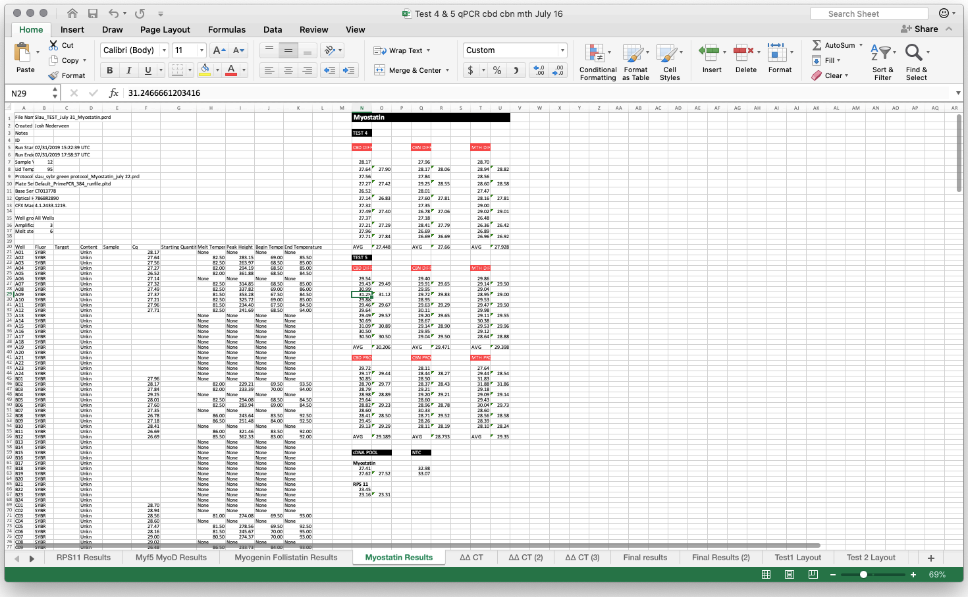

Test 4 & 5 qPCR cbd cbn mth July 16
RPS 11
RPS11 Results
Myf5 MyoD Results
Myogenin Follistatin Results
Myostatin Results
ΔΔ CT
ΔΔ CT (2)
ΔΔ CT (3)
Final results
Final Results (2)
Test1 Layout
Test 2 Layout


Test 4 & 5 qPCR cbd cbn mth July 16
Myf5
MyoD
RPS11 Results
Myf5 MyoD Results
Myogenin Follistatin Results
Myostatin Results
ΔΔ CT
ΔΔ CT (2)
ΔΔ CT (3)
Final results
Final Results (2)
Test1 Layout
Test 2 Layout

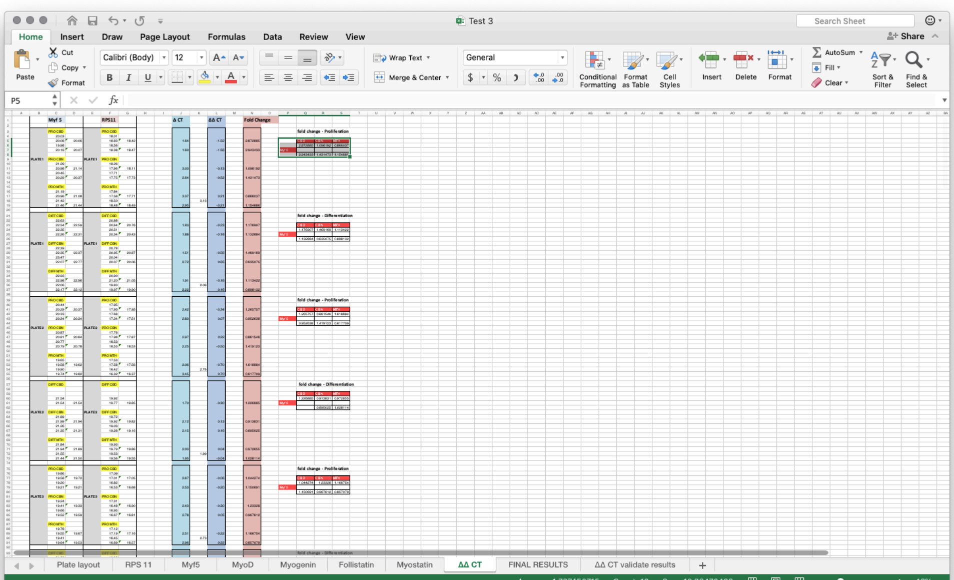

Test 4 & 5 qPCR cbd cbn mth July 16
RPS11 Results
Myf5 MyoD Results
Myogenin Follistatin Results
Myostatin Results
ΔΔ CT
ΔΔ CT (2)
ΔΔ CT (3)
Final results
Final Results (2)
Test1 Layout
Test 2 Layout

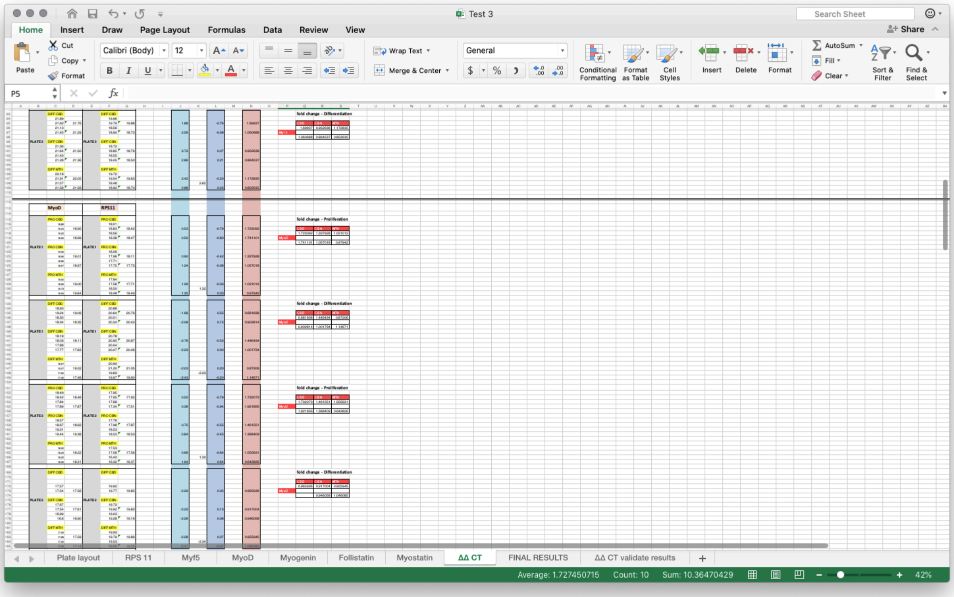

Test 4 & 5 qPCR cbd cbn mth July 16
Myogenin
Follistatin
RPS11 Results
Myf5 MyoD Results
Myogenin Follistatin Results
Myostatin Results
Final results
Final Results (2)
Test1 Layout
Test 2 Layout


Test 4 & 5 qPCR cbd cbn mth July 16
Follistatin
RPS11 Results
Myf5 MyoD Results
Myogenin Follistatin Results
Myostatin Results
Final results
Final Results (2)
Test1 Layout
Test 2 Layout



Real-Time PCR Analysis Fold Change (5uM) TRIAL 3




Test 3
Home Insert Draw Page Layout Formulas Data Review View
Plate layout RPS 11 Myf5 MyoD Myogenin Follistatin Myostatin ΔΔ CT FINAL RESULTS ΔΔ CT validate results
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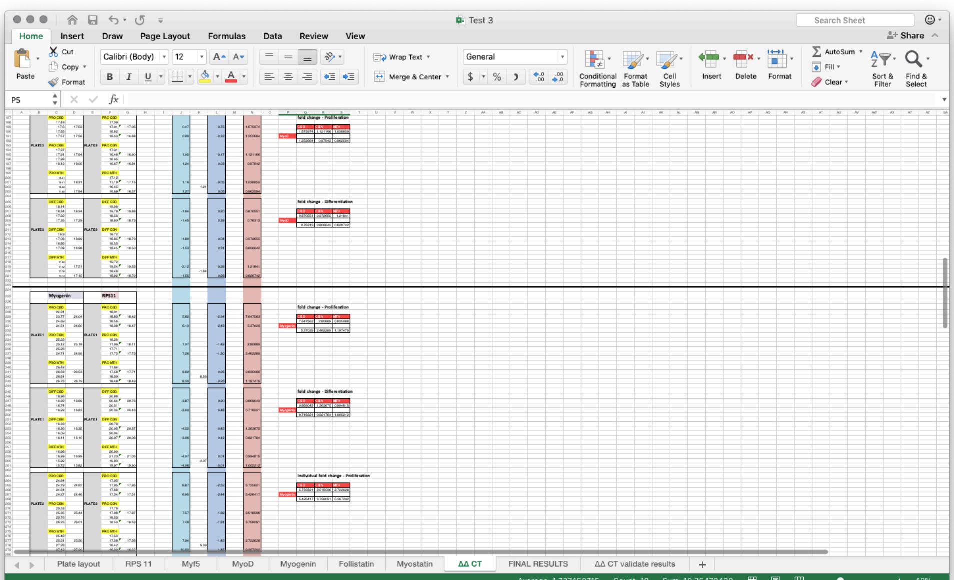
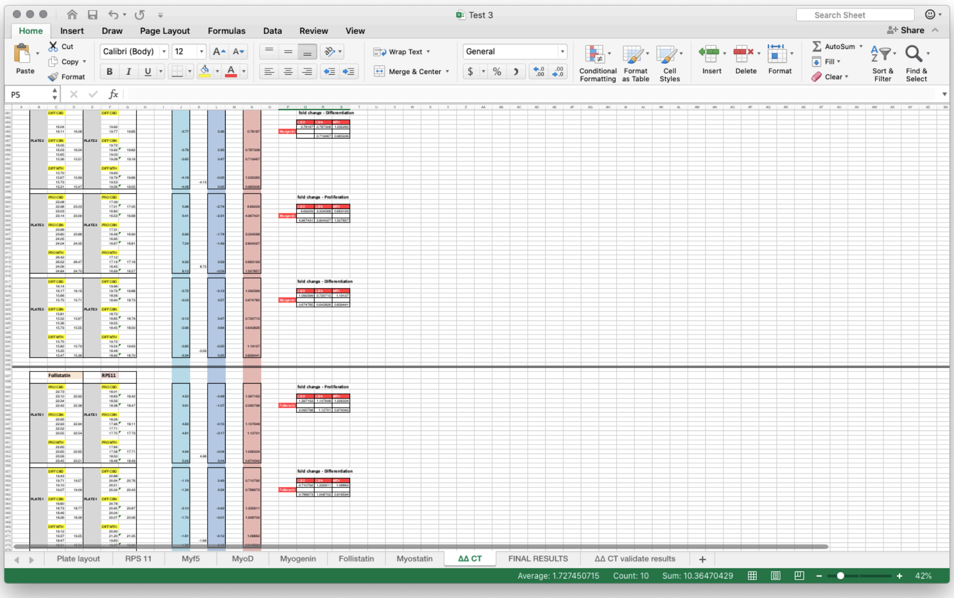
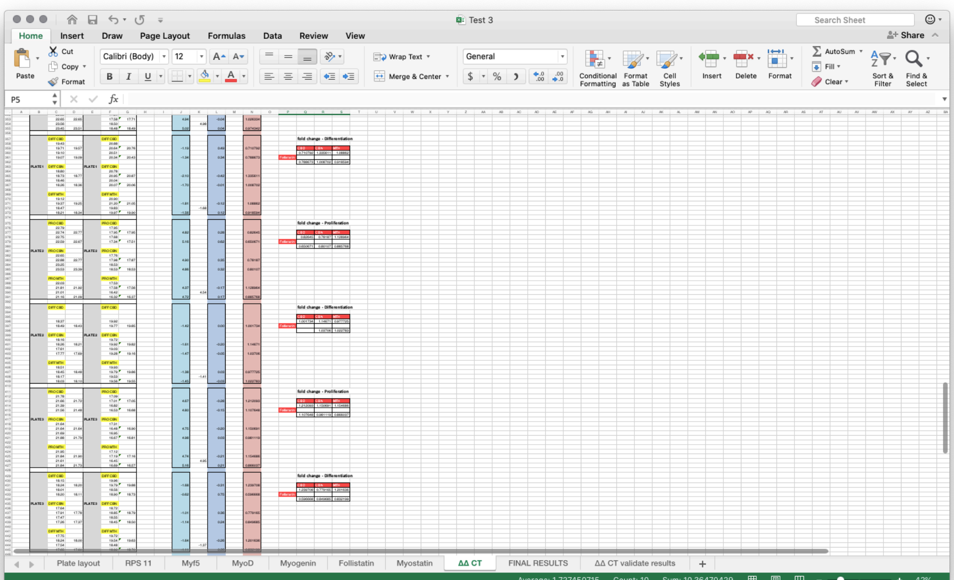
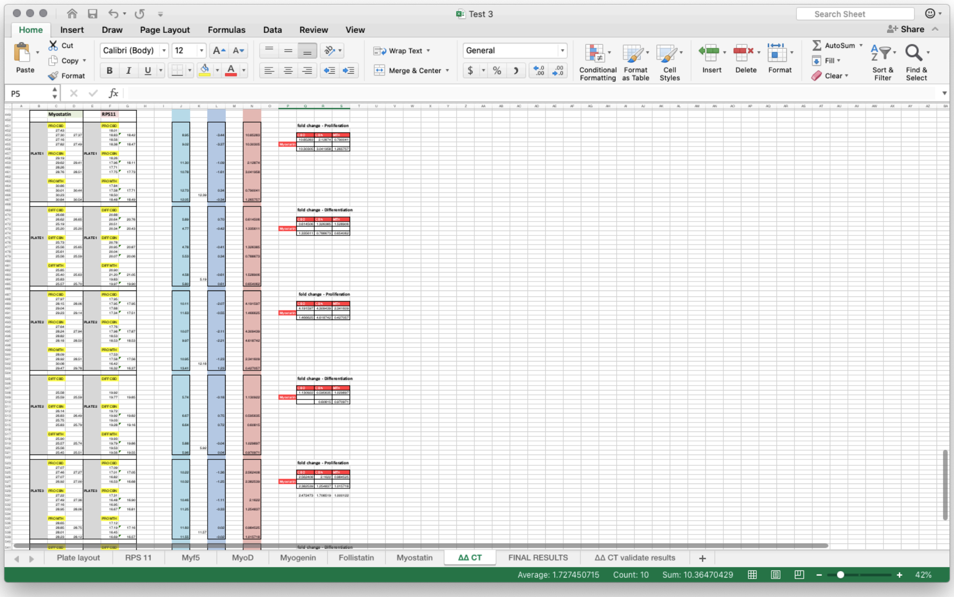
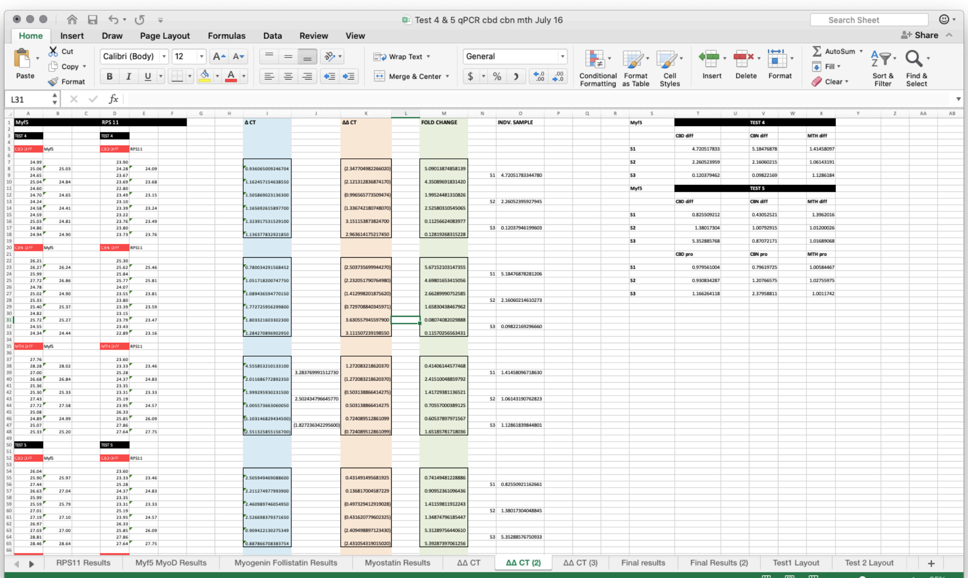

Test 3
Home Insert Draw Page Layout Formulas Data Review View
Plate layout RPS 11 Myf5 MyoD Myogenin Follistatin Myostatin ΔΔ CT FINAL RESULTS ΔΔ CT validate results
Average: 1.727450715 Count: 10 Sum: 10.36470429

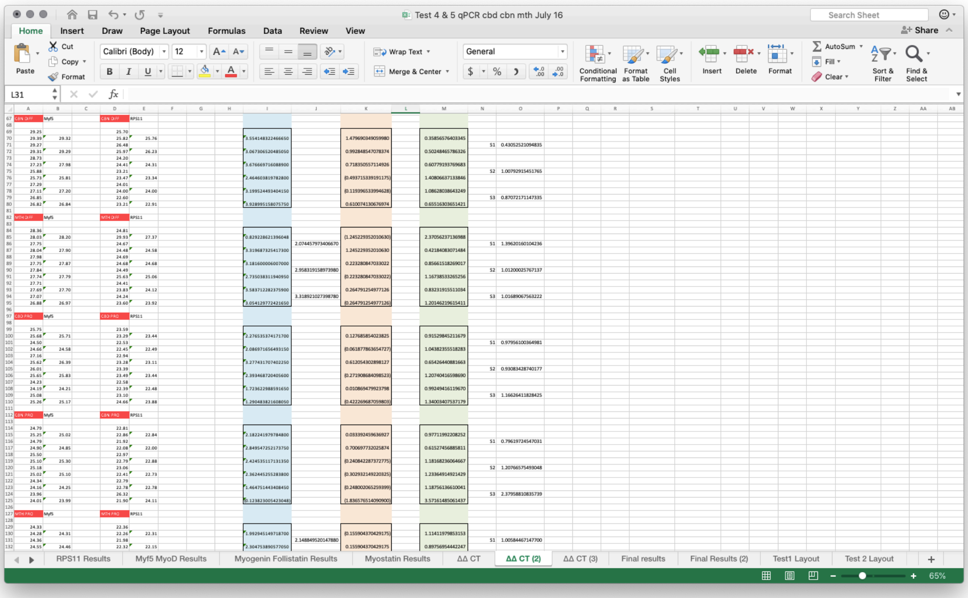

Test 3
Plate layout
RPS 11
Myf5
MyoD
Myogenin
Follistatin
Myostatin
ΔΔ CT
FINAL RESULTS
ΔΔ CT validate results
Average: 1.727450715
Count: 10
Sum: 10.36470429

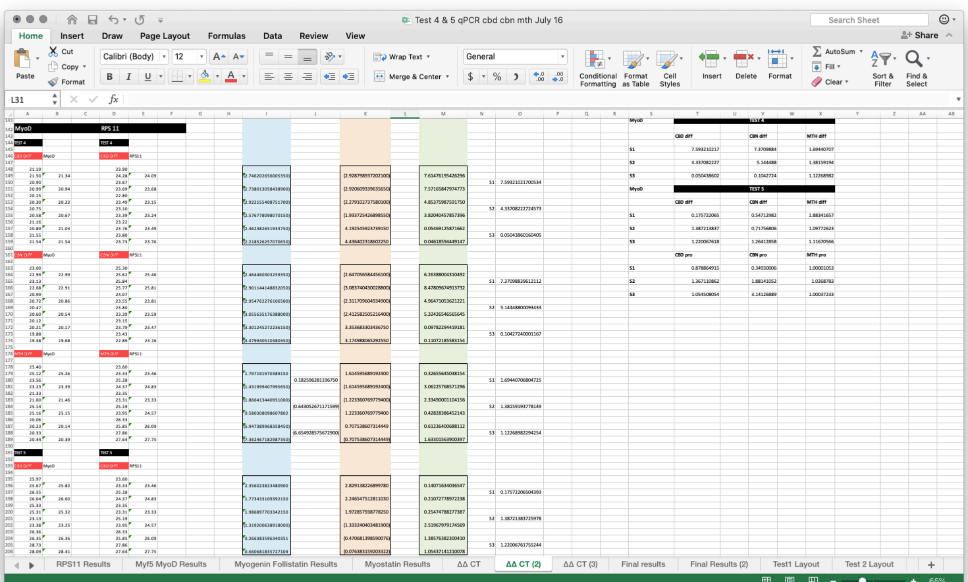

Test 3
Plate layout
RPS 11
Myf5
MyoD
Myogenin
Follistatin
Myostatin
ΔΔ CT
FINAL RESULTS
ΔΔ CT validate results
Average: 1.727450715
Count: 10
Sum: 10.36470429

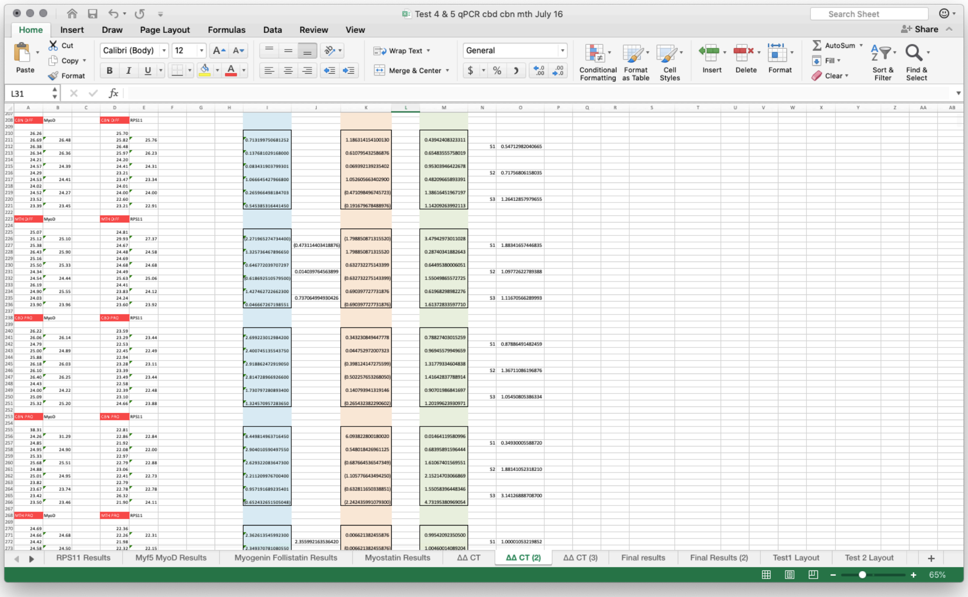


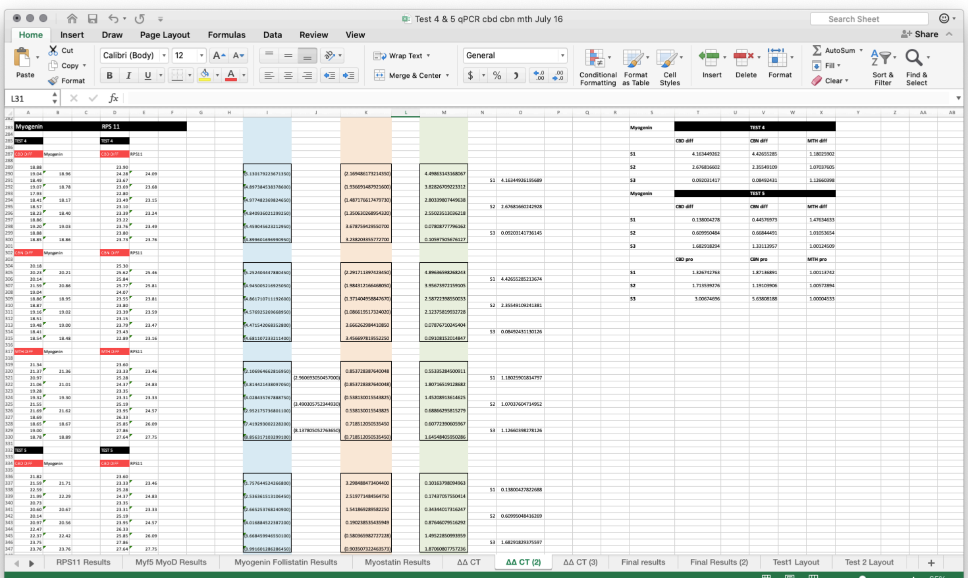
Real-Time PCR Analysis Fold Change (5uM) TRIAL 4 & 5

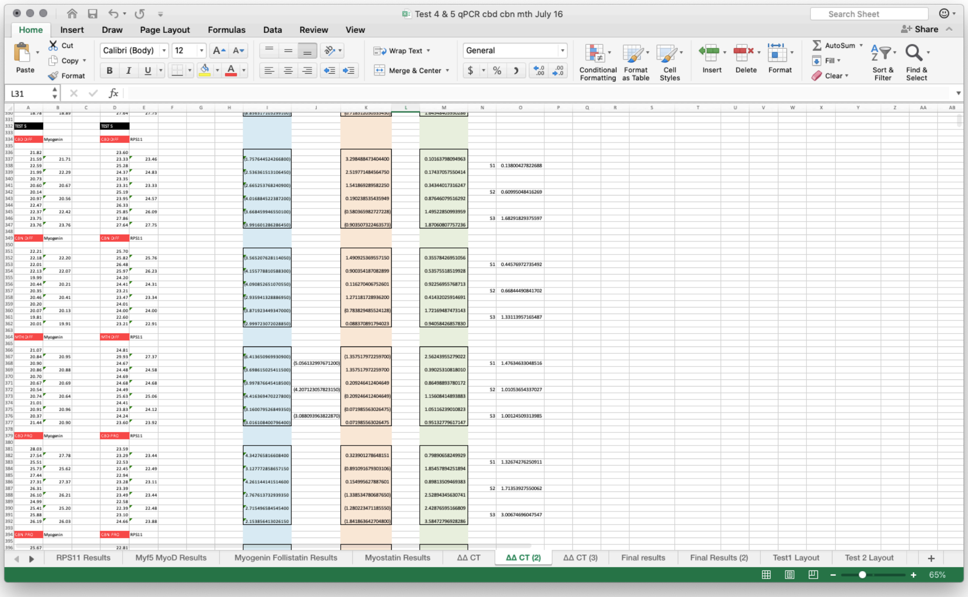


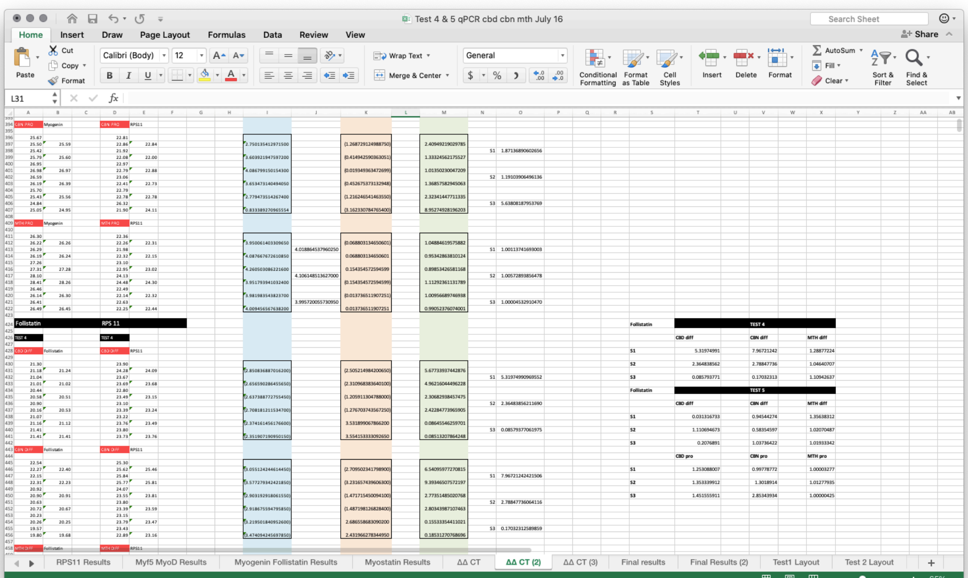

Test 4 & 5 qPCR cbd cbn mth July 16
RPS11 Results
Myf5 MyoD Results
Myogenin Follistatin Results
Myostatin Results
ΔΔ CT
ΔΔ CT (2)
ΔΔ CT (3)
Final results
Final Results (2)
Test1 Layout
Test 2 Layout

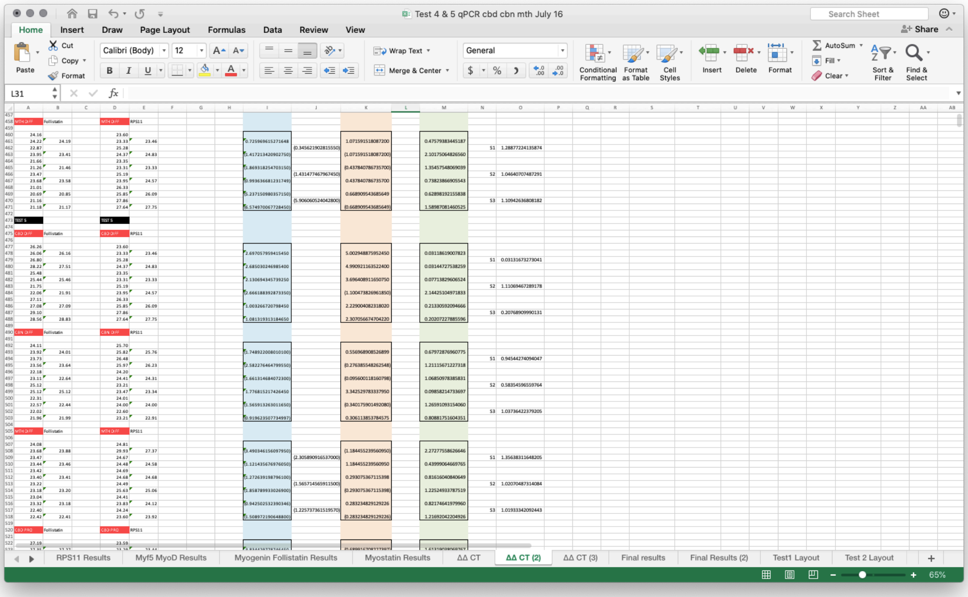

Test 4 & 5 qPCR cbd cbn mth July 16
RPS11 Results
Myf5 MyoD Results
Myogenin Follistatin Results
Myostatin Results
ΔΔ CT
ΔΔ CT (2)
ΔΔ CT (3)
Final results
Final Results (2)
Test1 Layout
Test 2 Layout

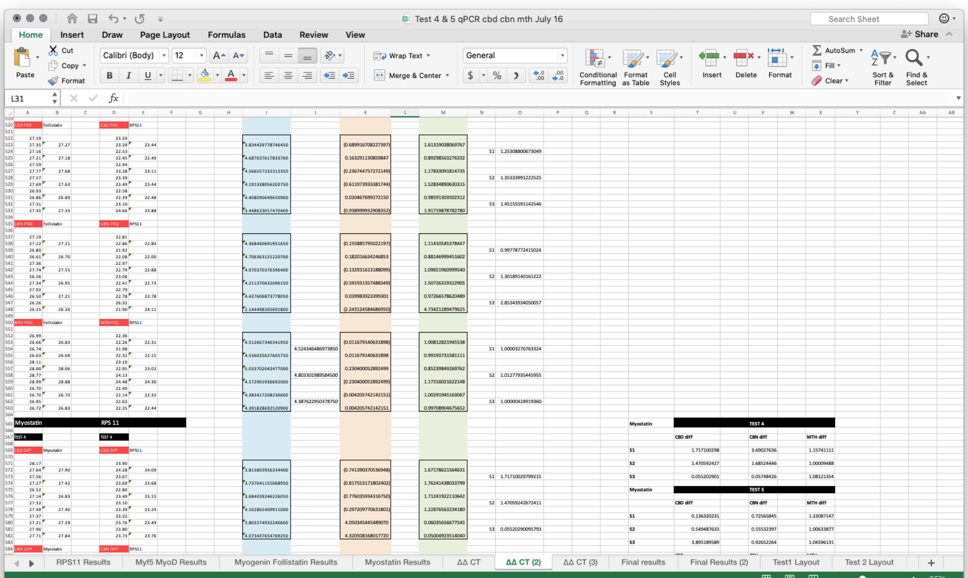

Test 4 & 5 qPCR cbd cbn mth July 16
RPS11 Results
Myf5 MyoD Results
Myogenin Follistatin Results
Myostatin Results
ΔΔ CT
ΔΔ CT (2)
ΔΔ CT (3)
Final results
Final Results (2)
Test1 Layout
Test 2 Layout

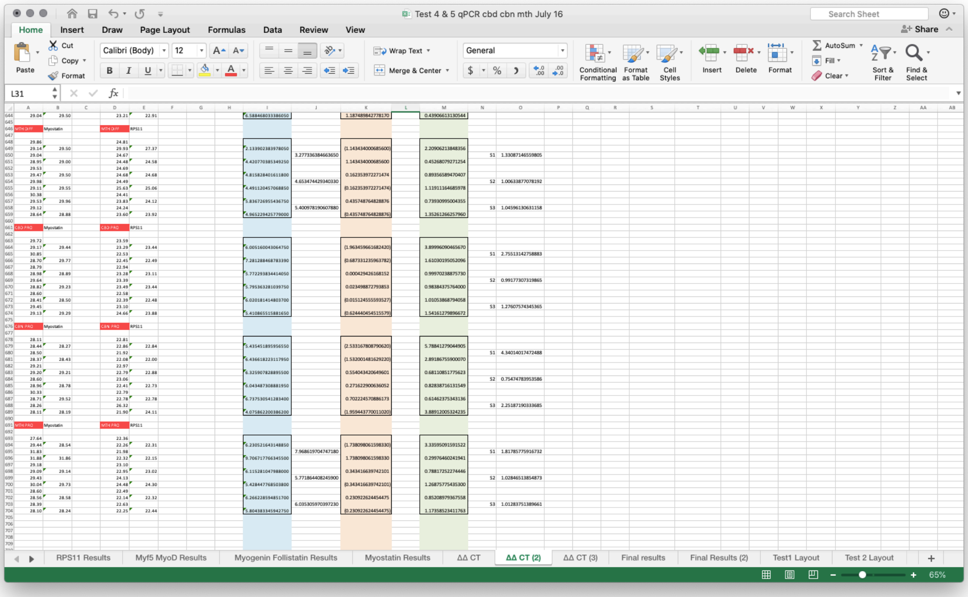

Test 4 & 5 qPCR cbd cbn mth July 16
RPS11 Results
Myf5 MyoD Results
Myogenin Follistatin Results
Myostatin Results
ΔΔ CT
ΔΔ CT (2)
ΔΔ CT (3)
Final results
Final Results (2)
Test1 Layout
Test 2 Layout

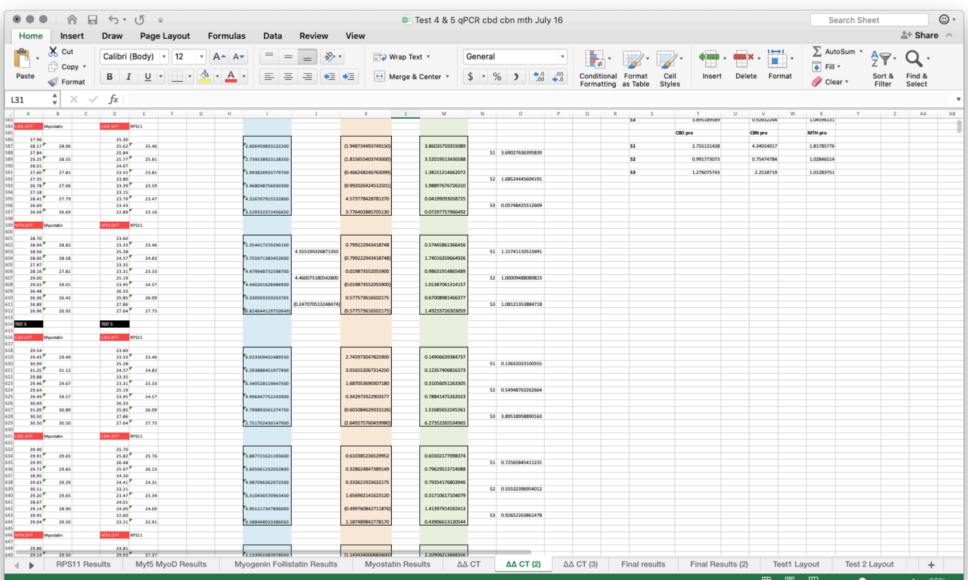

Test 4 & 5 qPCR cbd cbn mth July 16
RPS11 Results
Myf5 MyoD Results
Myogenin Follistatin Results
Myostatin Results
ΔΔ CT
ΔΔ CT (2)
ΔΔ CT (3)
Final results
Final Results (2)
Test1 Layout
Test 2 Layout

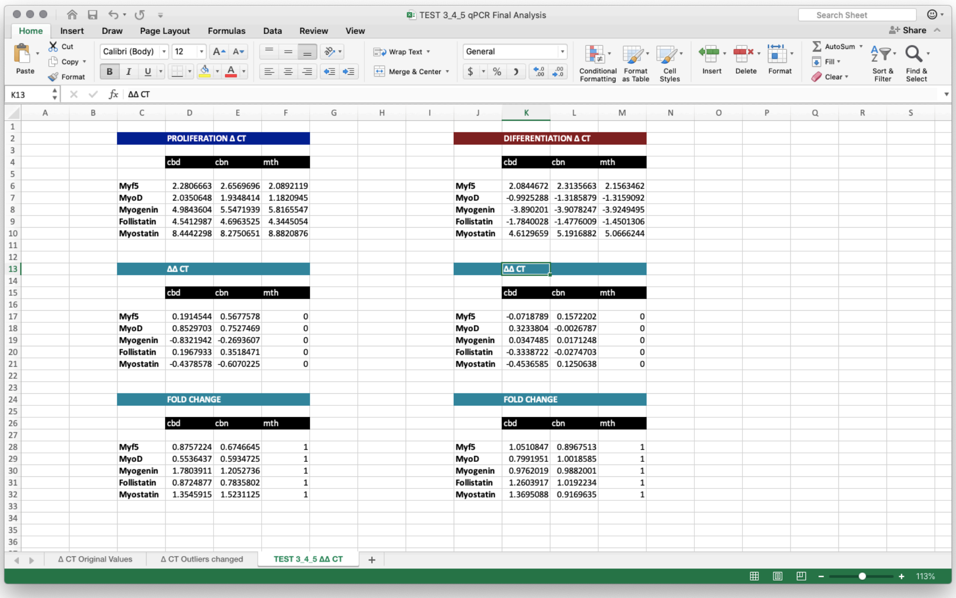

Test 4 & 5 qPCR cbd cbn mth July 16
RPS11 Results
Myf5 MyoD Results
Myogenin Follistatin Results
Myostatin Results
ΔΔ CT
ΔΔ CT (2)
ΔΔ CT (3)
Final results
Final Results (2)
Test1 Layout
Test 2 Layout


Test 4 & 5 qPCR cbd cbn mth July 16
RPS11 Results
Myf5 MyoD Results
Myogenin Follistatin Results
Myostatin Results
ΔΔ CT
ΔΔ CT (2)
ΔΔ CT (3)
Final results
Final Results (2)
Test1 Layout
Test 2 Layout


Test 4 & 5 qPCR cbd cbn mth July 16
RPS11 Results
Myf5 MyoD Results
Myogenin Follistatin Results
Myostatin Results
ΔΔ CT
ΔΔ CT (2)
ΔΔ CT (3)
Final results
Final Results (2)
Test1 Layout
Test 2 Layout


Test 4 & 5 qPCR cbd cbn mth July 16
RPS11 Results
Myf5 MyoD Results
Myogenin Follistatin Results
Myostatin Results
ΔΔ CT
ΔΔ CT (2)
ΔΔ CT (3)
Final results
Final Results (2)
Test1 Layout
Test 2 Layout


Test 4 & 5 qPCR cbd cbn mth July 16
RPS11 Results
Myf5 MyoD Results
Myogenin Follistatin Results
Myostatin Results
ΔΔ CT
ΔΔ CT (2)
ΔΔ CT (3)
Final results
Final Results (2)
Test1 Layout
Test 2 Layout



PROLIFERATION Δ CT	cbd	cbn	mth
Myf5	2.2806663	2.6569696	2.0892119
MyoD	2.0350648	1.9348414	1.1820945
Myogenin	4.9843604	5.5471939	5.8165547
Follistatin	4.5412987	4.6963525	4.3445054
Myostatin	8.4442298	8.2750651	8.8820876

DIFFERENTIATION Δ CT	cbd	cbn	mth
Myf5	2.0844672	2.3135663	2.1563462
MyoD	-0.9925288	-1.3185879	-1.3159092
Myogenin	-3.890201	-3.9078247	-3.9249495
Follistatin	-1.7840028	-1.4776009	-1.4501306
Myostatin	4.6129659	5.1916882	5.0666244

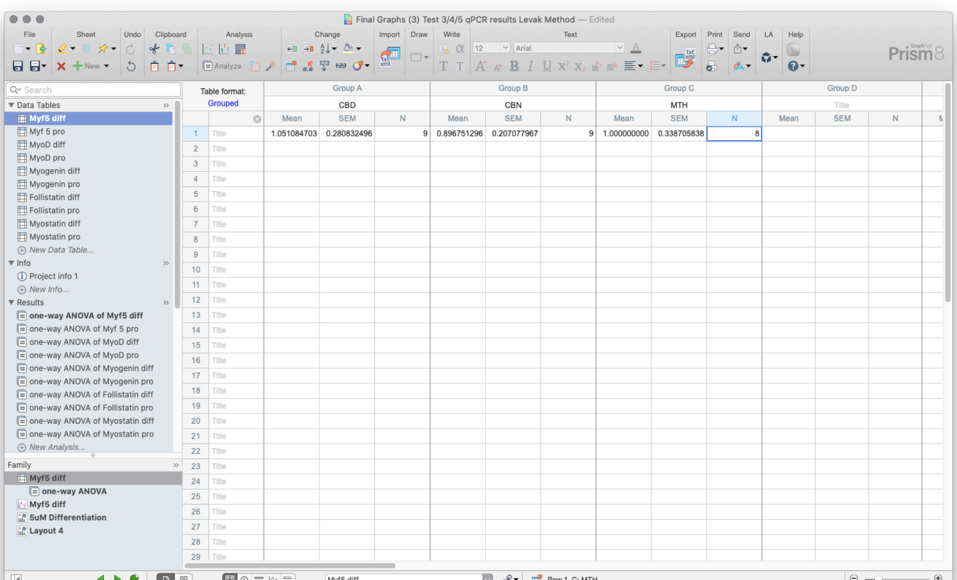
ΔΔ CT (Proliferation)	cbd	cbn	mth
Myf5	0.1914544	0.5677578	0
MyoD	0.8529703	0.7527469	0
Myogenin	-0.8321942	-0.2693607	0
Follistatin	0.1967933	0.3518471	0
Myostatin	-0.4378578	-0.6070225	0

ΔΔ CT (Differentiation)	cbd	cbn	mth
Myf5	-0.0718789	0.1572202	0
MyoD	0.3233804	-0.0026787	0
Myogenin	0.0347485	0.0171248	0
Follistatin	-0.3338722	-0.0274703	0
Myostatin	-0.4536585	0.1250638	0

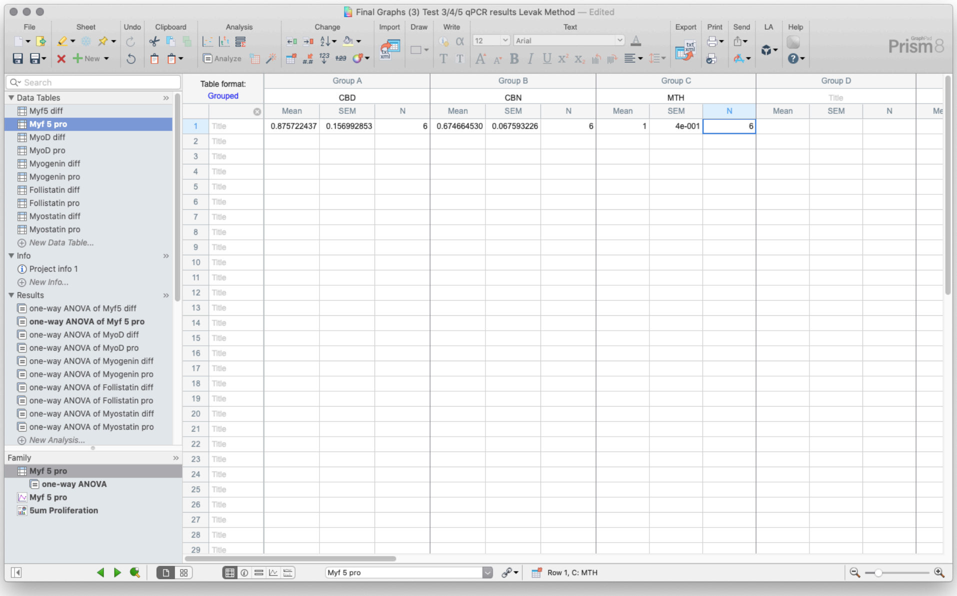
FOLD CHANGE (Proliferation)	cbd	cbn	mth
Myf5	0.8757224	0.6746645	1
MyoD	0.5536437	0.5934725	1
Myogenin	1.7803911	1.2052736	1
Follistatin	0.8724877	0.7835802	1
Myostatin	1.3545915	1.5231125	1

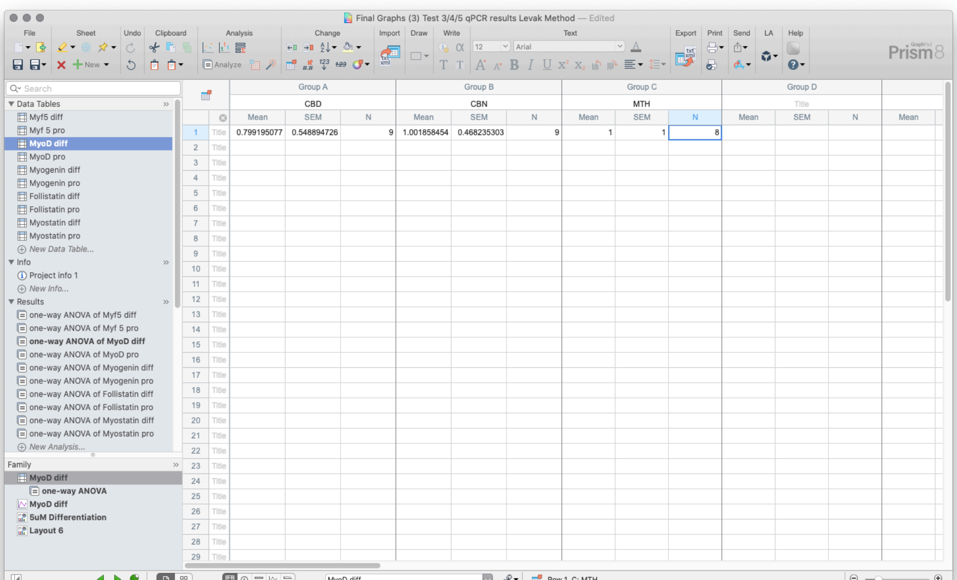
FOLD CHANGE (Differentiation)	cbd	cbn	mth
Myf5	1.0510847	0.8967513	1
MyoD	0.7991951	1.0018585	1
Myogenin	0.9762019	0.9882001	1
Follistatin	1.2603917	1.0192234	1
Myostatin	1.3695088	0.9169635	1

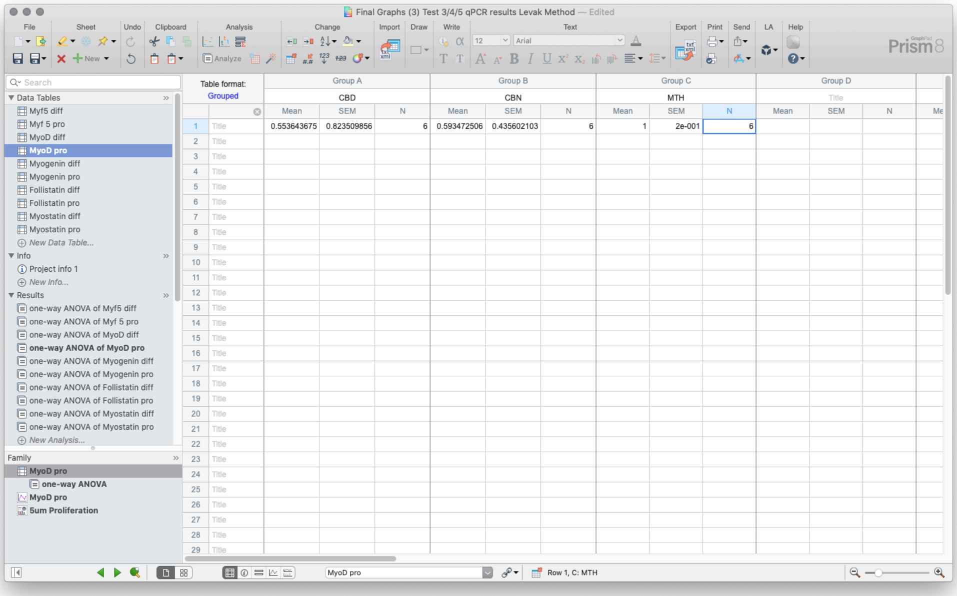
Real-Time PCR Analysis on PRSIM (5uM)

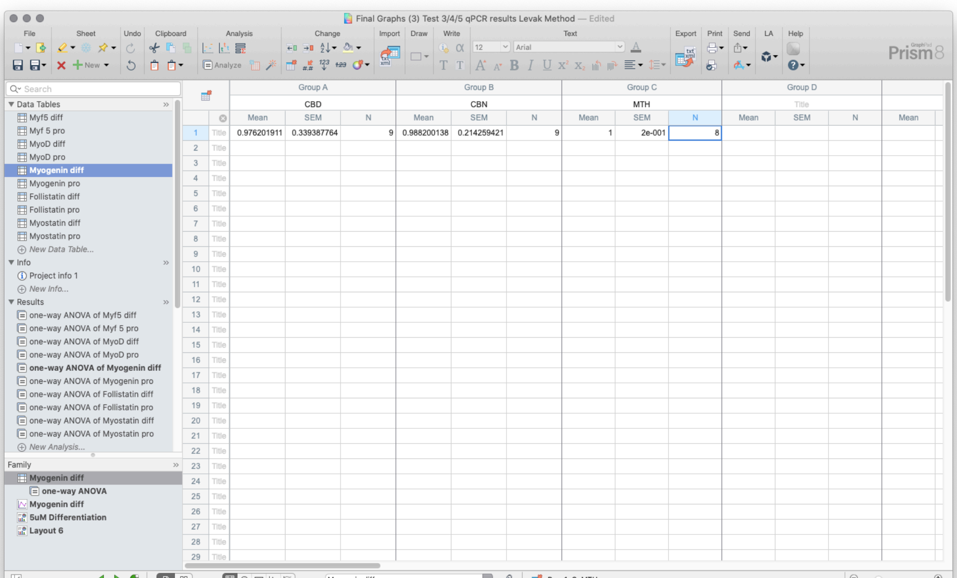


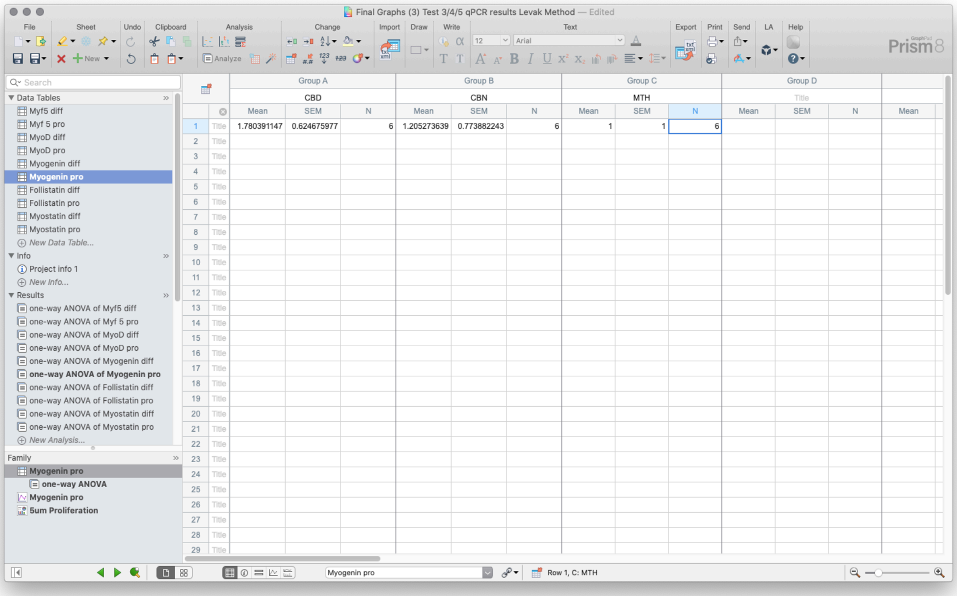
	Group A: CBD			Group B: CBN			Group C: MTH		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	1.051084703	0.280832496	9	0.896751296	0.207077967	9	1.000000000	0.338705838	8

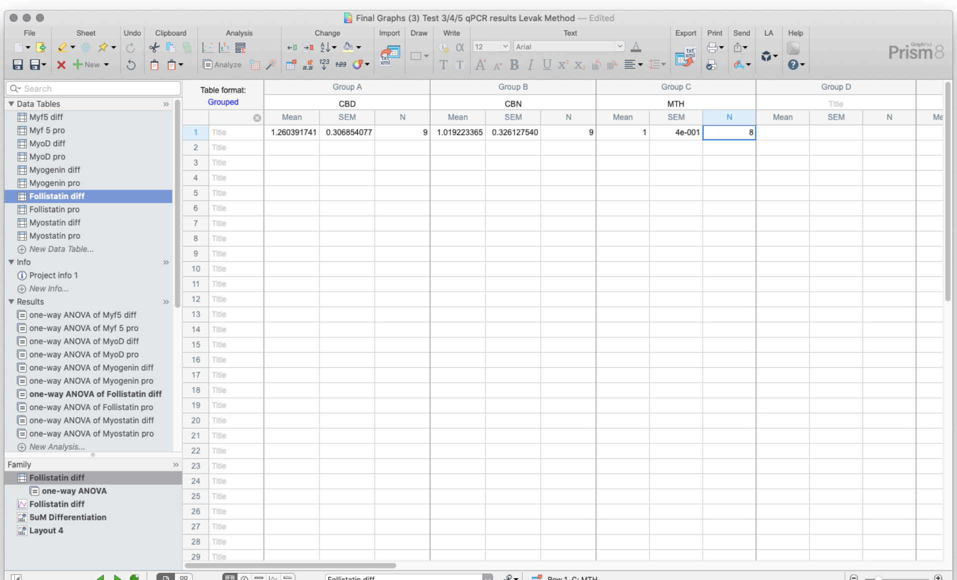

Final Graphs (3) Test 3/4/5 qPCR results Levak Method — Edited
Table format: Grouped
Group A
CBD
Group B
CBN
Group C
MTH
Group D
Mean SEM N
1 Title 0.875722437 0.156992853 6 0.674664530 0.067593226 6 1 4e-001 6
Data Tables
Myf5 diff
Myf 5 pro
MyoD diff
MyoD pro
Myogenin diff
Myogenin pro
Follistatin diff
Follistatin pro
Myostatin diff
Myostatin pro
Info
Project info 1
Results
Family
Myf 5 pro
one-way ANOVA
5um Proliferation
Row 1, C: MTH

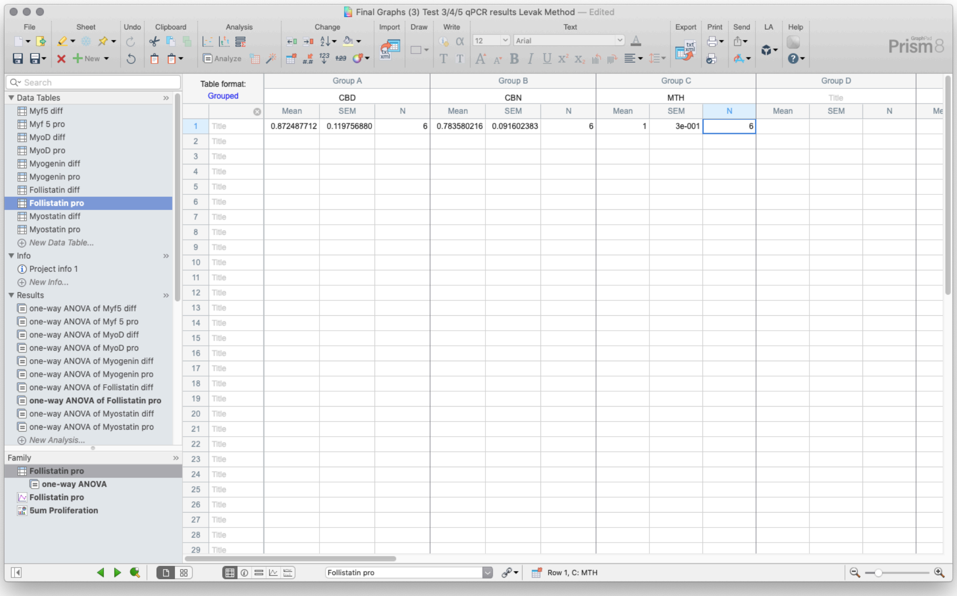

Final Graphs (3) Test 3/4/5 qPCR results Levak Method — Edited
Group A
CBD
Group B
CBN
Group C
MTH
Group D
Mean SEM N
1 Title 0.799195077 0.548894726 9 1.001858454 0.468235303 9 1 1 8
Family
MyoD diff
one-way ANOVA
5uM Differentiation
Layout 6
Row 1, C: MTH

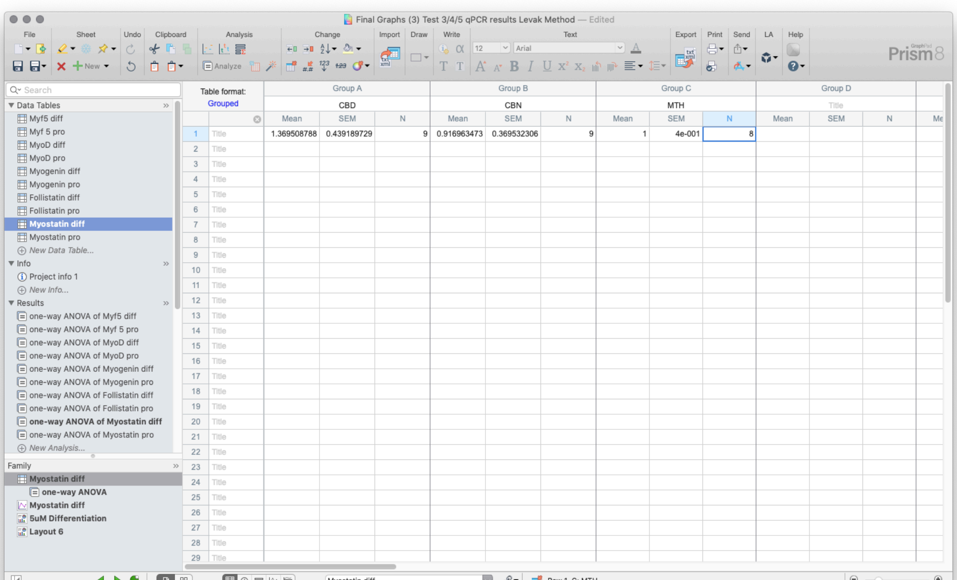

Final Graphs (3) Test 3/4/5 qPCR results Levak Method — Edited
Table format:
Grouped
Group A
Group B
Group C
Group D
CBD
CBN
MTH
Mean
SEM
N
0.553643675
0.823509856
6
0.593472506
0.435602103
6
1
2e-001
6
Data Tables
Myf5 diff
Myf 5 pro
MyoD diff
MyoD pro
Myogenin diff
Myogenin pro
Follistatin diff
Follistatin pro
Myostatin diff
Myostatin pro
Info
Project info 1
Results
one-way ANOVA of Myf5 diff
one-way ANOVA of Myf 5 pro
one-way ANOVA of MyoD diff
one-way ANOVA of MyoD pro
one-way ANOVA of Myogenin diff
one-way ANOVA of Myogenin pro
one-way ANOVA of Follistatin diff
one-way ANOVA of Follistatin pro
one-way ANOVA of Myostatin diff
one-way ANOVA of Myostatin pro
Family
MyoD pro
one-way ANOVA
MyoD pro
5um Proliferation
Row 1, C: MTH

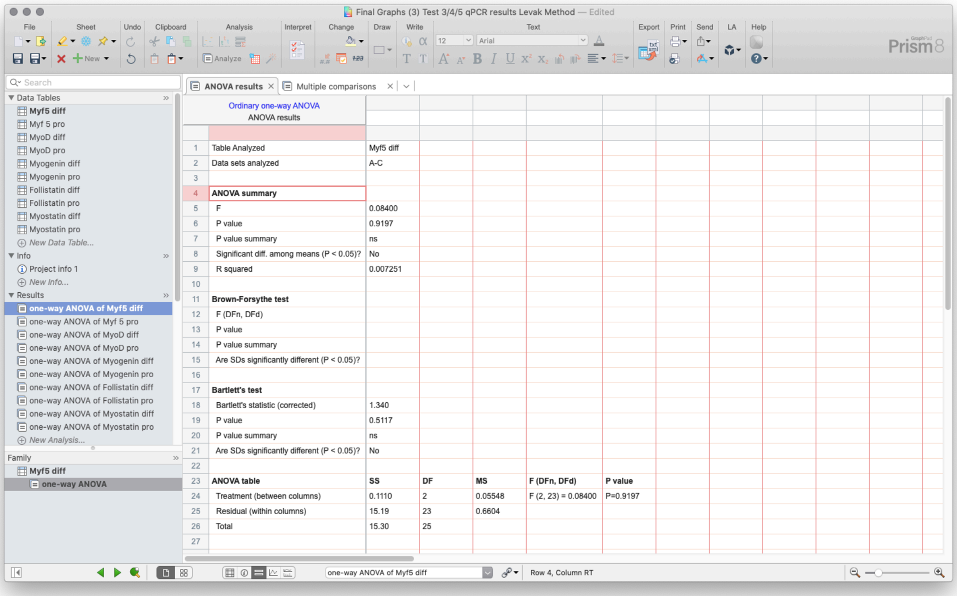

Final Graphs (3) Test 3/4/5 qPCR results Levak Method — Edited
Group A
Group B
Group C
Group D
CBD
CBN
MTH
Mean
SEM
N
0.976201911
0.339387764
9
0.988200138
0.214259421
9
1
2e-001
8
Data Tables
Myf5 diff
Myf 5 pro
MyoD diff
MyoD pro
Myogenin diff
Myogenin pro
Follistatin diff
Follistatin pro
Myostatin diff
Myostatin pro
Info
Project info 1
Results
one-way ANOVA of Myf5 diff
one-way ANOVA of Myf 5 pro
one-way ANOVA of MyoD diff
one-way ANOVA of MyoD pro
one-way ANOVA of Myogenin diff
one-way ANOVA of Myogenin pro
one-way ANOVA of Follistatin diff
one-way ANOVA of Follistatin pro
one-way ANOVA of Myostatin diff
one-way ANOVA of Myostatin pro
Family
Myogenin diff
one-way ANOVA
Myogenin diff
5uM Differentiation
Layout 6
Row 1, C: MTH


Final Graphs (3) Test 3/4/5 qPCR results Levak Method — Edited
Group A
CBD
Mean SEM N
1.780391147 0.624675977 6
Group B
CBN
Mean SEM N
1.205273639 0.773882243 6
Group C
MTH
Mean SEM N
1 1 6
Group D
Myogenin pro
Row 1, C: MTH


Final Graphs (3) Test 3/4/5 qPCR results Levak Method — Edited
Table format:
Grouped
Group A
CBD
Mean SEM N
1.260391741 0.306854077 9
Group B
CBN
Mean SEM N
1.019223365 0.326127540 9
Group C
MTH
Mean SEM N
1 4e-001 8
Group D
Follistatin diff
Row 1, C: MTH

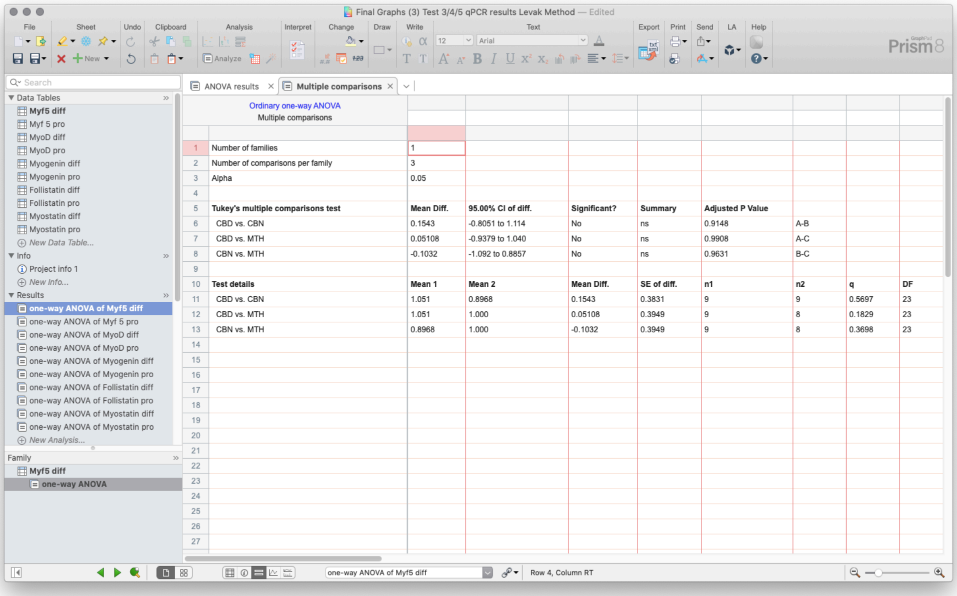

Final Graphs (3) Test 3/4/5 qPCR results Levak Method — Edited
Table format: Grouped
Group A
CBD
Group B
CBN
Group C
MTH
Group D
Mean
SEM
N
0.872487712
0.119756880
6
0.783580216
0.091602383
6
1
3e-001
6
Follistatin pro
Row 1, C: MTH


Final Graphs (3) Test 3/4/5 qPCR results Levak Method — Edited
Table format: Grouped
Group A
CBD
Group B
CBN
Group C
MTH
Group D
Mean
SEM
N
1.369508788
0.439189729
9
0.916963473
0.369532306
9
1
4e-001
8
Myostatin diff
Row 1, C: MTH

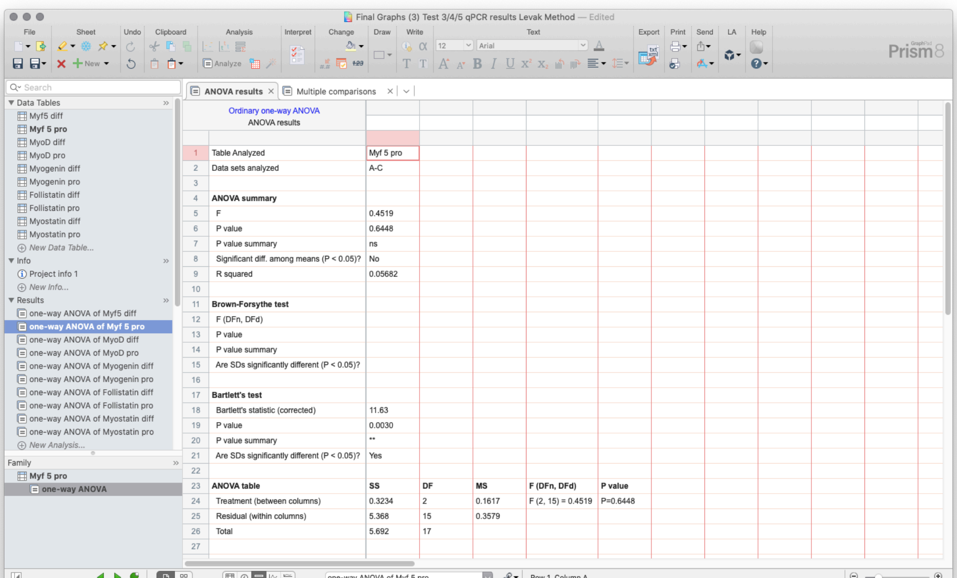


Ordinary one-way ANOVA
ANOVA results

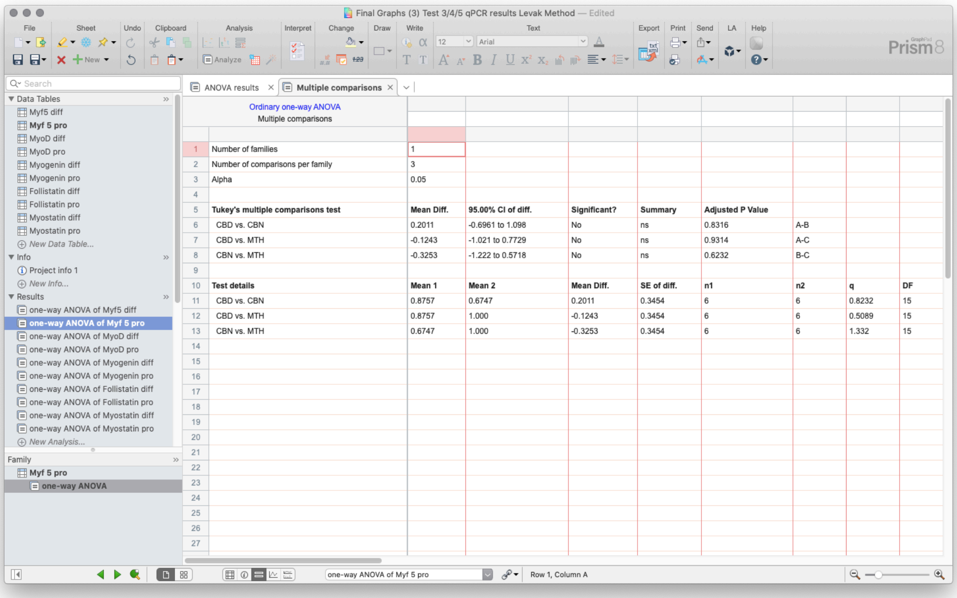
1	Table Analyzed	Myf5 diff				
2	Data sets analyzed	A-C				
3						
4	**ANOVA summary**					
5	F	0.08400				
6	P value	0.9197				
7	P value summary	ns				
8	Significant diff. among means (P < 0.05)?	No				
9	R squared	0.007251				
10						
11	**Brown-Forsythe test**					
12	F (DFn, DFd)					
13	P value					
14	P value summary					
15	Are SDs significantly different (P < 0.05)?					
16						
17	**Bartlett's test**					
18	Bartlett's statistic (corrected)	1.340				
19	P value	0.5117				
20	P value summary	ns				
21	Are SDs significantly different (P < 0.05)?	No				
22						
23	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
24	Treatment (between columns)	0.1110	2	0.05548	F (2, 23) = 0.08400	P=0.9197
25	Residual (within columns)	15.19	23	0.6604		
26	Total	15.30	25			
27						



Ordinary one-way ANOVA Multiple comparisons								
Number of families	1							
Number of comparisons per family	3							
Alpha	0.05							
Tukey's multiple comparisons test	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**			
CBD vs. CBN	0.1543	-0.8051 to 1.114	No	ns	0.9148	A-B		
CBD vs. MTH	0.05108	-0.9379 to 1.040	No	ns	0.9908	A-C		
CBN vs. MTH	-0.1032	-1.092 to 0.8857	No	ns	0.9631	B-C		
Test details	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
CBD vs. CBN	1.051	0.8968	0.1543	0.3831	9	9	0.5697	23
CBD vs. MTH	1.051	1.000	0.05108	0.3949	9	8	0.1829	23
CBN vs. MTH	0.8968	1.000	-0.1032	0.3949	9	8	0.3698	23



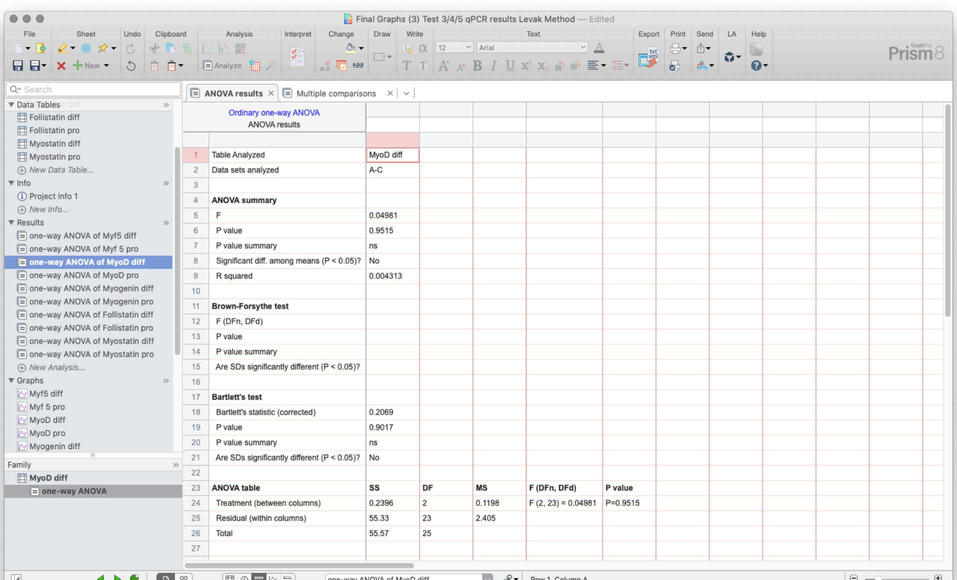
Ordinary one-way ANOVA ANOVA results					
Table Analyzed	Myf 5 pro				
Data sets analyzed	A-C				
ANOVA summary					
F	0.4519				
P value	0.6448				
P value summary	ns				
Significant diff. among means (P < 0.05)?	No				
R squared	0.05682				
Brown-Forsythe test					
F (DFn, DFd)					
P value					
P value summary					
Are SDs significantly different (P < 0.05)?					
Bartlett's test					
Bartlett's statistic (corrected)	11.63				
P value	0.0030				
P value summary	**				
Are SDs significantly different (P < 0.05)?	Yes				
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Treatment (between columns)	0.3234	2	0.1617	F (2, 15) = 0.4519	P=0.6448
Residual (within columns)	5.368	15	0.3579		
Total	5.692	17			



Final Graphs (3) Test 3/4/5 qPCR results Levak Method — Edited

Ordinary one-way ANOVA
Multiple comparisons

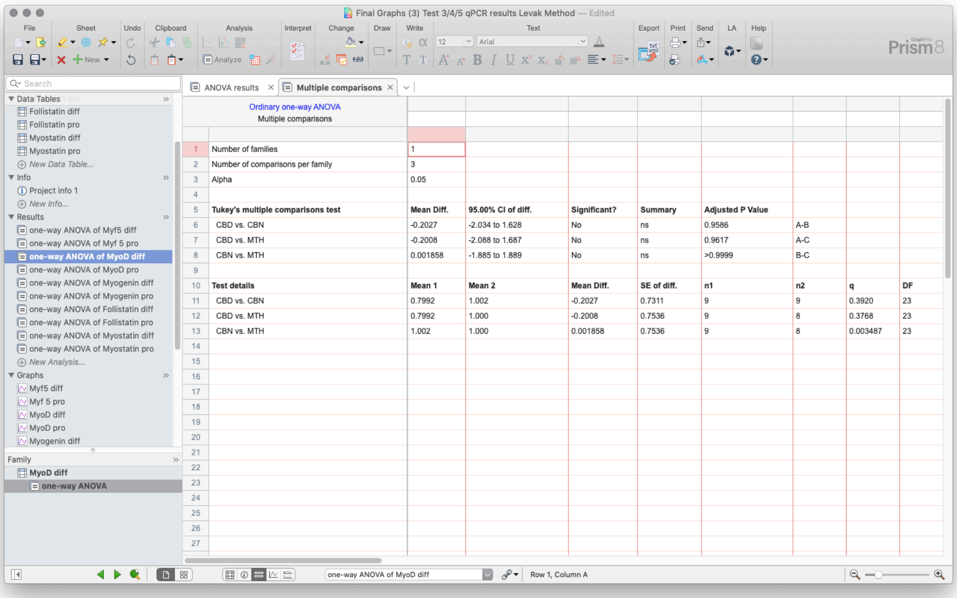
1	Number of families	1								
2	Number of comparisons per family	3								
3	Alpha	0.05								
4										
5	**Tukey's multiple comparisons test**	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**				
6	CBD vs. CBN	0.2011	-0.6961 to 1.098	No	ns	0.8316	A-B			
7	CBD vs. MTH	-0.1243	-1.021 to 0.7729	No	ns	0.9314	A-C			
8	CBN vs. MTH	-0.3253	-1.222 to 0.5718	No	ns	0.6232	B-C			
9										
10	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**	
11	CBD vs. CBN	0.8757	0.6747	0.2011	0.3454	6	6	0.8232	15	
12	CBD vs. MTH	0.8757	1.000	-0.1243	0.3454	6	6	0.5089	15	
13	CBN vs. MTH	0.6747	1.000	-0.3253	0.3454	6	6	1.332	15	

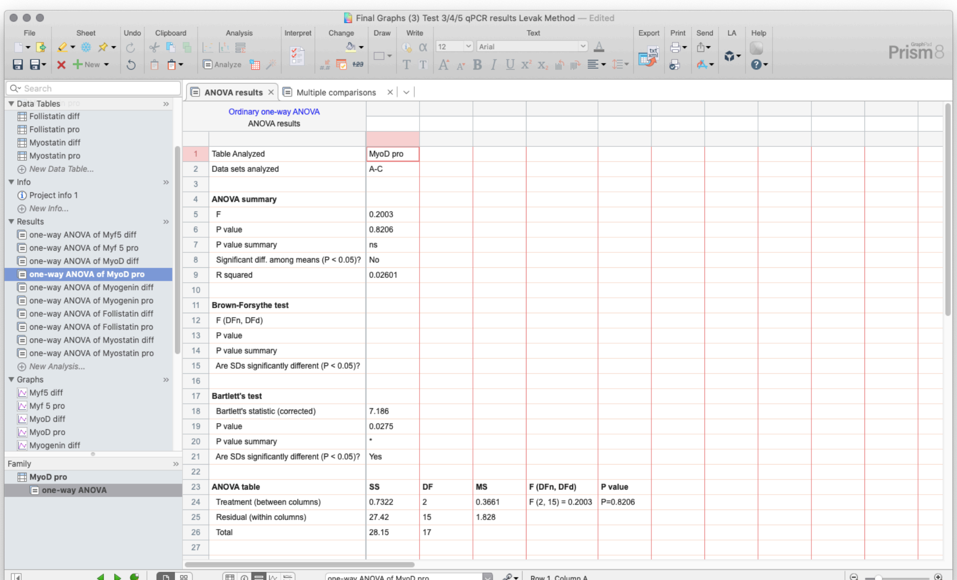


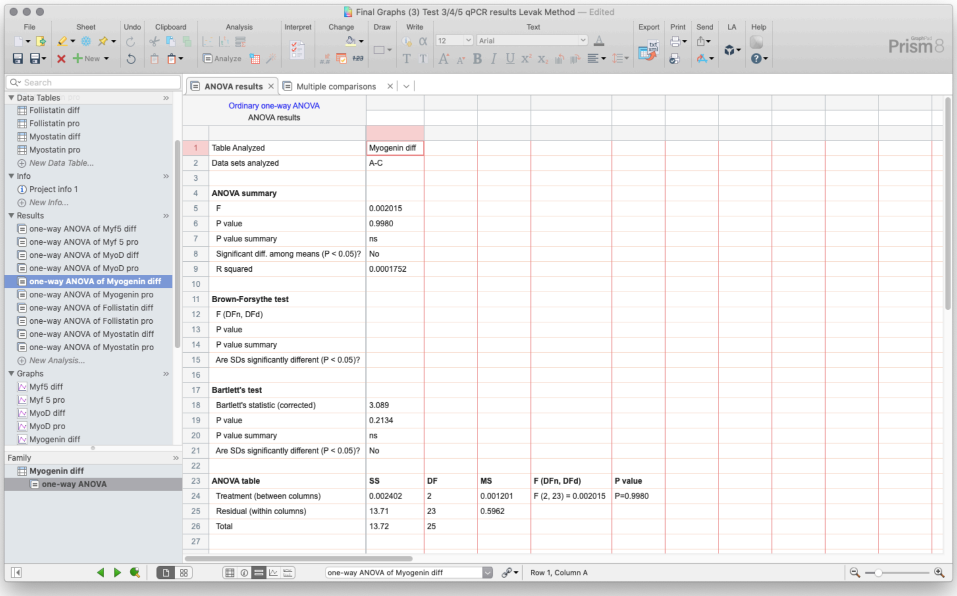
Final Graphs (3) Test 3/4/5 qPCR results Levak Method — Edited

Ordinary one-way ANOVA
ANOVA results

1	Table Analyzed	MyoD diff				
2	Data sets analyzed	A-C				
3						
4	**ANOVA summary**					
5	F	0.04981				
6	P value	0.9515				
7	P value summary	ns				
8	Significant diff. among means (P < 0.05)?	No				
9	R squared	0.004313				
10						
11	**Brown-Forsythe test**					
12	F (DFn, DFd)					
13	P value					
14	P value summary					
15	Are SDs significantly different (P < 0.05)?					
16						
17	**Bartlett's test**					
18	Bartlett's statistic (corrected)	0.2069				
19	P value	0.9017				
20	P value summary	ns				
21	Are SDs significantly different (P < 0.05)?	No				
22						
23	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
24	Treatment (between columns)	0.2396	2	0.1198	F (2, 23) = 0.04981	P=0.9515
25	Residual (within columns)	55.33	23	2.405		
26	Total	55.57	25			

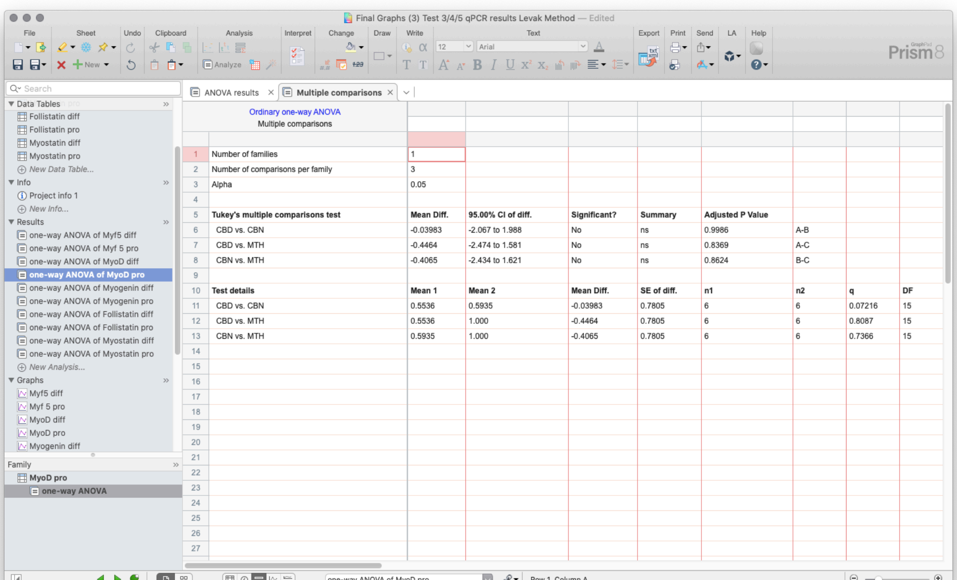

Final Graphs (3) Test 3/4/5 qPCR results Levak Method — Edited
ANOVA results
Multiple comparisons
Ordinary one-way ANOVA
Multiple comparisons
Number of families 1
Number of comparisons per family 3
Alpha 0.05
Tukey's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Significant? | Summary | Adjusted P Value
CBD vs. CBN | -0.2027 | -2.034 to 1.628 | No | ns | 0.9586 | A-B
CBD vs. MTH | -0.2008 | -2.088 to 1.687 | No | ns | 0.9617 | A-C
CBN vs. MTH | 0.001858 | -1.885 to 1.889 | No | ns | >0.9999 | B-C
Test details | Mean 1 | Mean 2 | Mean Diff. | SE of diff. | n1 | n2 | q | DF
CBD vs. CBN | 0.7992 | 1.002 | -0.2027 | 0.7311 | 9 | 9 | 0.3920 | 23
CBD vs. MTH | 0.7992 | 1.000 | -0.2008 | 0.7536 | 9 | 8 | 0.3768 | 23
CBN vs. MTH | 1.002 | 1.000 | 0.001858 | 0.7536 | 9 | 8 | 0.003487 | 23
one-way ANOVA of MyoD diff
Row 1, Column A


Final Graphs (3) Test 3/4/5 qPCR results Levak Method — Edited
ANOVA results
Multiple comparisons
Ordinary one-way ANOVA
ANOVA results
Table Analyzed MyoD pro
Data sets analyzed A-C
ANOVA summary
F 0.2003
P value 0.8206
P value summary ns
Significant diff. among means (P < 0.05)? No
R squared 0.02601
Brown-Forsythe test
F (DFn, DFd)
P value
P value summary
Are SDs significantly different (P < 0.05)?
Bartlett's test
Bartlett's statistic (corrected) 7.186
P value 0.0275
P value summary *
Are SDs significantly different (P < 0.05)? Yes
ANOVA table | SS | DF | MS | F (DFn, DFd) | P value
Treatment (between columns) | 0.7322 | 2 | 0.3661 | F (2, 15) = 0.2003 | P=0.8206
Residual (within columns) | 27.42 | 15 | 1.828
Total | 28.15 | 17
one-way ANOVA of MyoD pro
Row 1, Column A



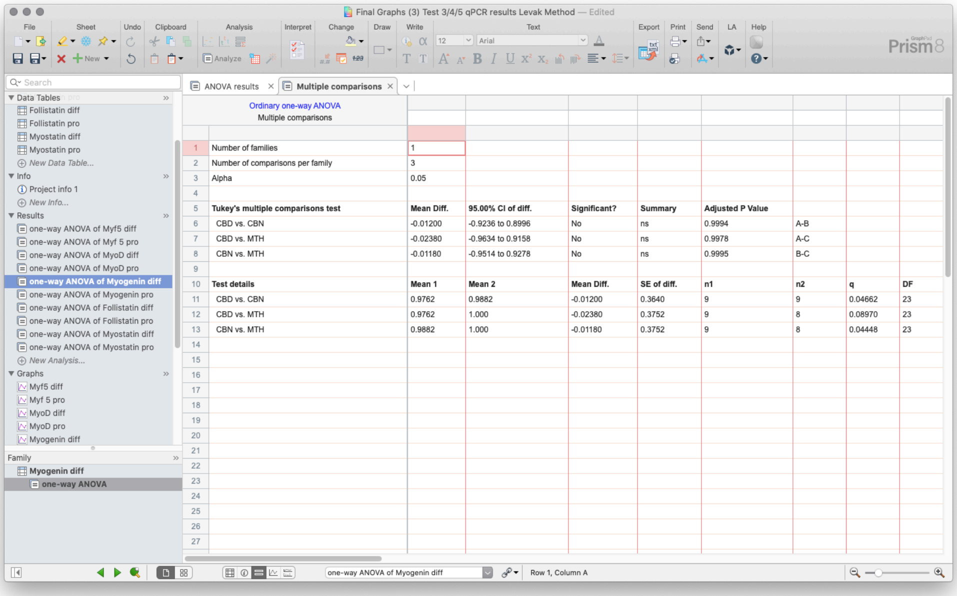
Ordinary one-way ANOVA
ANOVA results

1	Table Analyzed	Myogenin diff				
2	Data sets analyzed	A-C				
3						
4	**ANOVA summary**					
5	F	0.002015				
6	P value	0.9980				
7	P value summary	ns				
8	Significant diff. among means (P < 0.05)?	No				
9	R squared	0.0001752				
10						
11	**Brown-Forsythe test**					
12	F (DFn, DFd)					
13	P value					
14	P value summary					
15	Are SDs significantly different (P < 0.05)?					
16						
17	**Bartlett's test**					
18	Bartlett's statistic (corrected)	3.089				
19	P value	0.2134				
20	P value summary	ns				
21	Are SDs significantly different (P < 0.05)?	No				
22						
23	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
24	Treatment (between columns)	0.002402	2	0.001201	F (2, 23) = 0.002015	P=0.9980
25	Residual (within columns)	13.71	23	0.5962		
26	Total	13.72	25			
27						



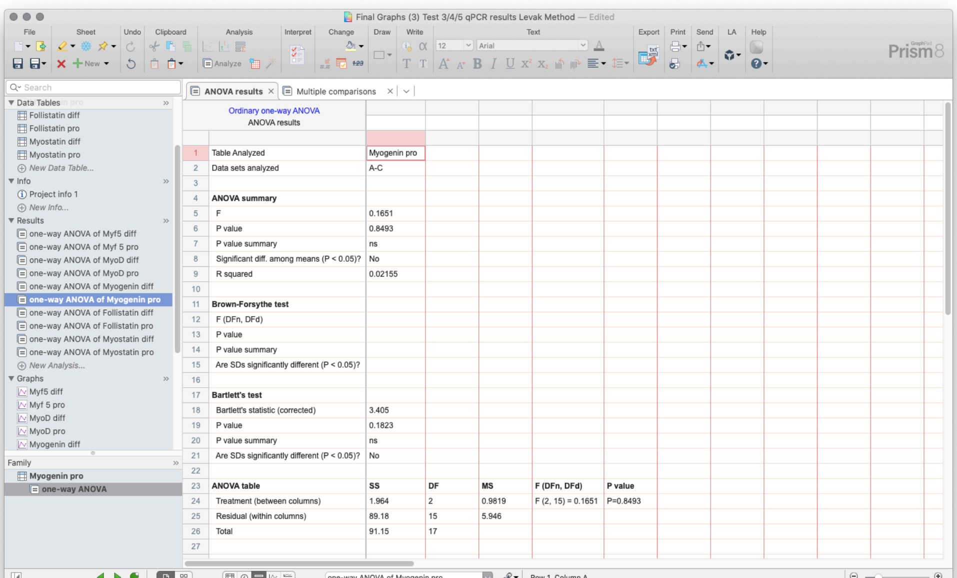
Ordinary one-way ANOVA
Multiple comparisons

1	Number of families	1							
2	Number of comparisons per family	3							
3	Alpha	0.05							
4									
5	**Tukey's multiple comparisons test**	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**			
6	CBD vs. CBN	-0.03983	-2.067 to 1.988	No	ns	0.9986	A-B		
7	CBD vs. MTH	-0.4464	-2.474 to 1.581	No	ns	0.8369	A-C		
8	CBN vs. MTH	-0.4065	-2.434 to 1.621	No	ns	0.8624	B-C		
9									
10	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
11	CBD vs. CBN	0.5536	0.5935	-0.03983	0.7805	6	6	0.07216	15
12	CBD vs. MTH	0.5536	1.000	-0.4464	0.7805	6	6	0.8087	15
13	CBN vs. MTH	0.5935	1.000	-0.4065	0.7805	6	6	0.7366	15



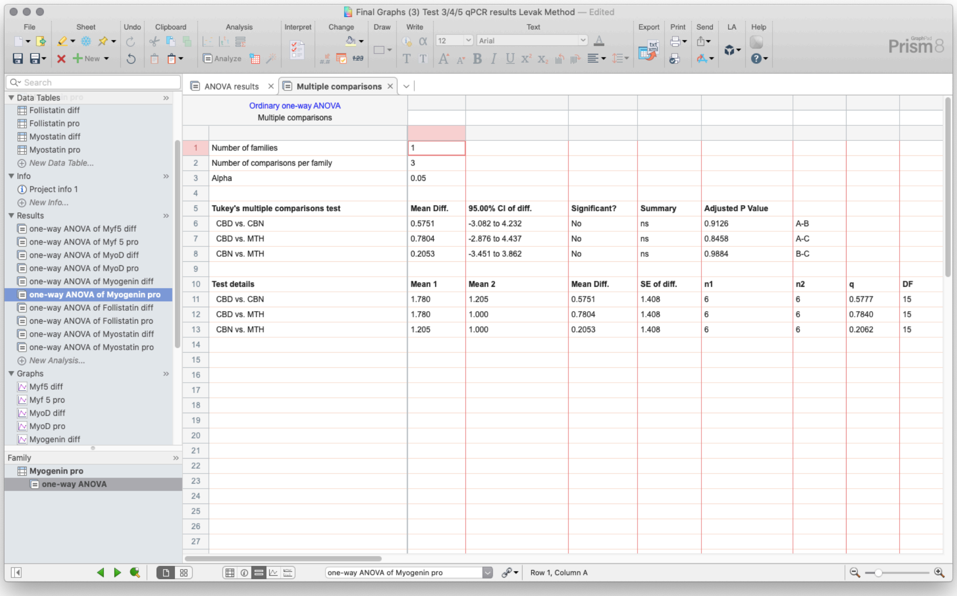
Ordinary one-way ANOVA
Multiple comparisons

1	Number of families	1							
2	Number of comparisons per family	3							
3	Alpha	0.05							
4									
5	**Tukey's multiple comparisons test**	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**			
6	CBD vs. CBN	-0.01200	-0.9236 to 0.8996	No	ns	0.9994	A-B		
7	CBD vs. MTH	-0.02380	-0.9634 to 0.9158	No	ns	0.9978	A-C		
8	CBN vs. MTH	-0.01180	-0.9514 to 0.9278	No	ns	0.9995	B-C		
9									
10	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
11	CBD vs. CBN	0.9762	0.9882	-0.01200	0.3640	9	9	0.04662	23
12	CBD vs. MTH	0.9762	1.000	-0.02380	0.3752	9	8	0.08970	23
13	CBN vs. MTH	0.9882	1.000	-0.01180	0.3752	9	8	0.04448	23



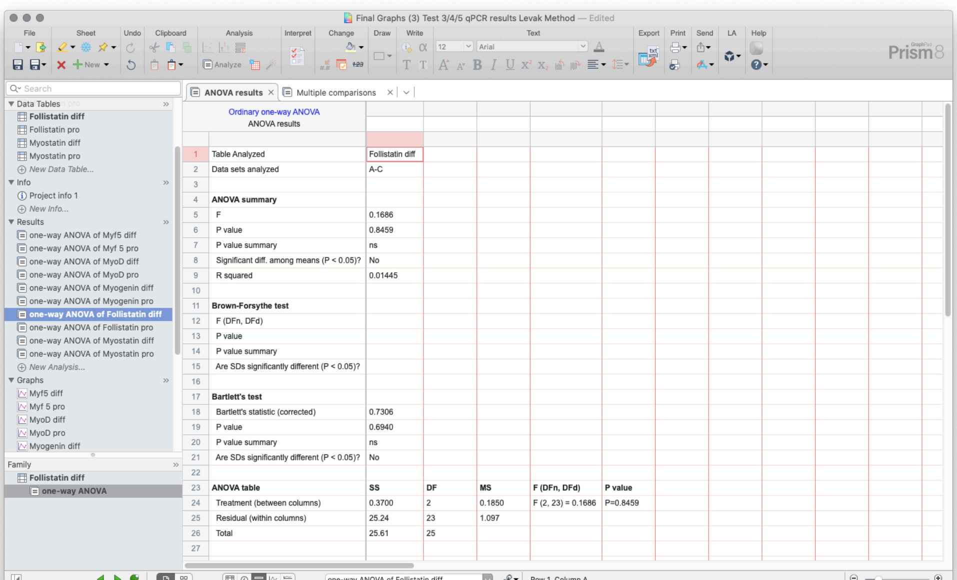
Ordinary one-way ANOVA
ANOVA results

1	Table Analyzed	Myogenin pro				
2	Data sets analyzed	A-C				
3						
4	**ANOVA summary**					
5	F	0.1651				
6	P value	0.8493				
7	P value summary	ns				
8	Significant diff. among means (P < 0.05)?	No				
9	R squared	0.02155				
10						
11	**Brown-Forsythe test**					
12	F (DFn, DFd)					
13	P value					
14	P value summary					
15	Are SDs significantly different (P < 0.05)?					
16						
17	**Bartlett's test**					
18	Bartlett's statistic (corrected)	3.405				
19	P value	0.1823				
20	P value summary	ns				
21	Are SDs significantly different (P < 0.05)?	No				
22						
23	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
24	Treatment (between columns)	1.964	2	0.9819	F (2, 15) = 0.1651	P=0.8493
25	Residual (within columns)	89.18	15	5.946		
26	Total	91.15	17			



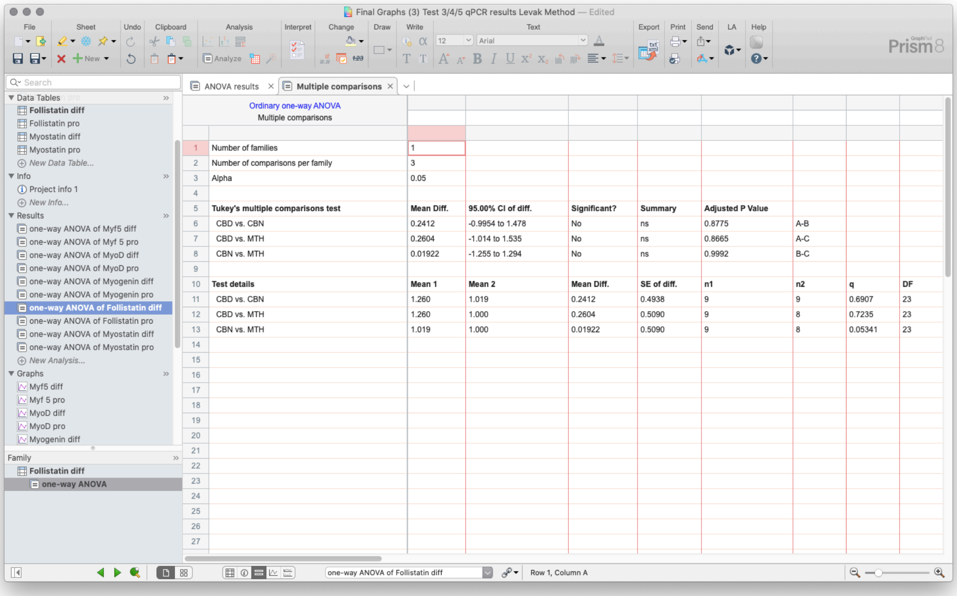
Ordinary one-way ANOVA
Multiple comparisons

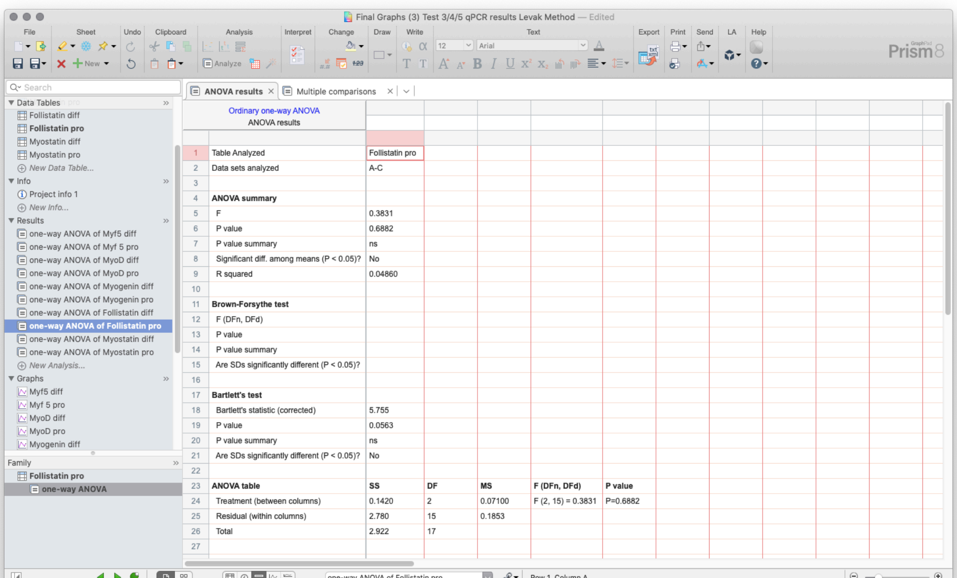
1	Number of families	1							
2	Number of comparisons per family	3							
3	Alpha	0.05							
4									
5	**Tukey's multiple comparisons test**	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**			
6	CBD vs. CBN	0.5751	-3.082 to 4.232	No	ns	0.9126	A-B		
7	CBD vs. MTH	0.7804	-2.876 to 4.437	No	ns	0.8458	A-C		
8	CBN vs. MTH	0.2053	-3.451 to 3.862	No	ns	0.9884	B-C		
9									
10	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
11	CBD vs. CBN	1.780	1.205	0.5751	1.408	6	6	0.5777	15
12	CBD vs. MTH	1.780	1.000	0.7804	1.408	6	6	0.7840	15
13	CBN vs. MTH	1.205	1.000	0.2053	1.408	6	6	0.2062	15

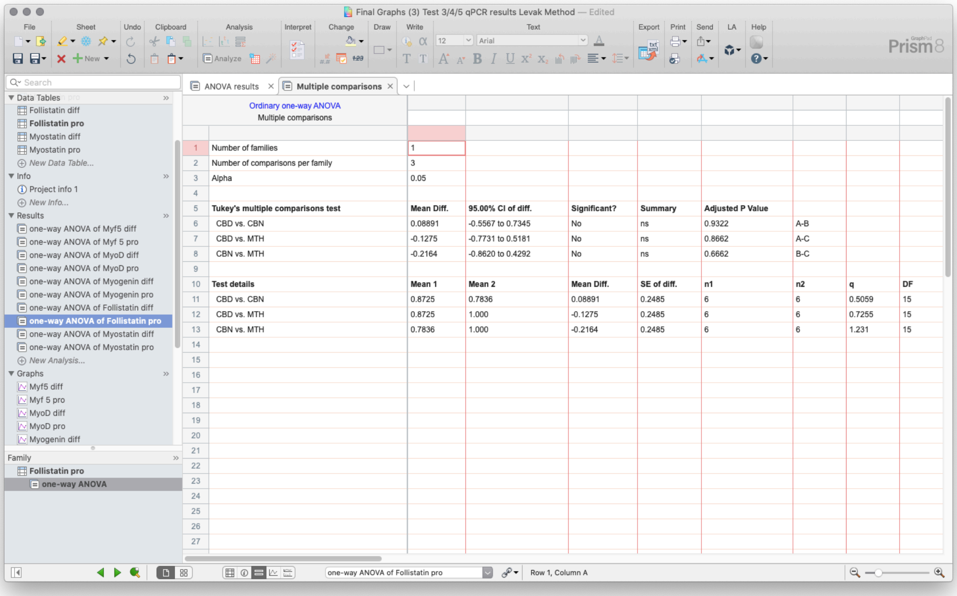


Ordinary one-way ANOVA
ANOVA results

1	Table Analyzed	Follistatin diff				
2	Data sets analyzed	A-C				
3						
4	**ANOVA summary**					
5	F	0.1686				
6	P value	0.8459				
7	P value summary	ns				
8	Significant diff. among means (P < 0.05)?	No				
9	R squared	0.01445				
10						
11	**Brown-Forsythe test**					
12	F (DFn, DFd)					
13	P value					
14	P value summary					
15	Are SDs significantly different (P < 0.05)?					
16						
17	**Bartlett's test**					
18	Bartlett's statistic (corrected)	0.7306				
19	P value	0.6940				
20	P value summary	ns				
21	Are SDs significantly different (P < 0.05)?	No				
22						
23	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
24	Treatment (between columns)	0.3700	2	0.1850	F (2, 23) = 0.1686	P=0.8459
25	Residual (within columns)	25.24	23	1.097		
26	Total	25.61	25			


Final Graphs (3) Test 3/4/5 qPCR results Levak Method — Edited
ANOVA results
Multiple comparisons
Ordinary one-way ANOVA
Multiple comparisons
Number of families 1
Number of comparisons per family 3
Alpha 0.05
Tukey's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Significant? | Summary | Adjusted P Value
CBD vs. CBN | 0.2412 | -0.9954 to 1.478 | No | ns | 0.8775 | A-B
CBD vs. MTH | 0.2604 | -1.014 to 1.535 | No | ns | 0.8665 | A-C
CBN vs. MTH | 0.01922 | -1.255 to 1.294 | No | ns | 0.9992 | B-C
Test details | Mean 1 | Mean 2 | Mean Diff. | SE of diff. | n1 | n2 | q | DF
CBD vs. CBN | 1.260 | 1.019 | 0.2412 | 0.4938 | 9 | 9 | 0.6907 | 23
CBD vs. MTH | 1.260 | 1.000 | 0.2604 | 0.5090 | 9 | 8 | 0.7235 | 23
CBN vs. MTH | 1.019 | 1.000 | 0.01922 | 0.5090 | 9 | 8 | 0.05341 | 23
one-way ANOVA of Follistatin diff
Row 1, Column A

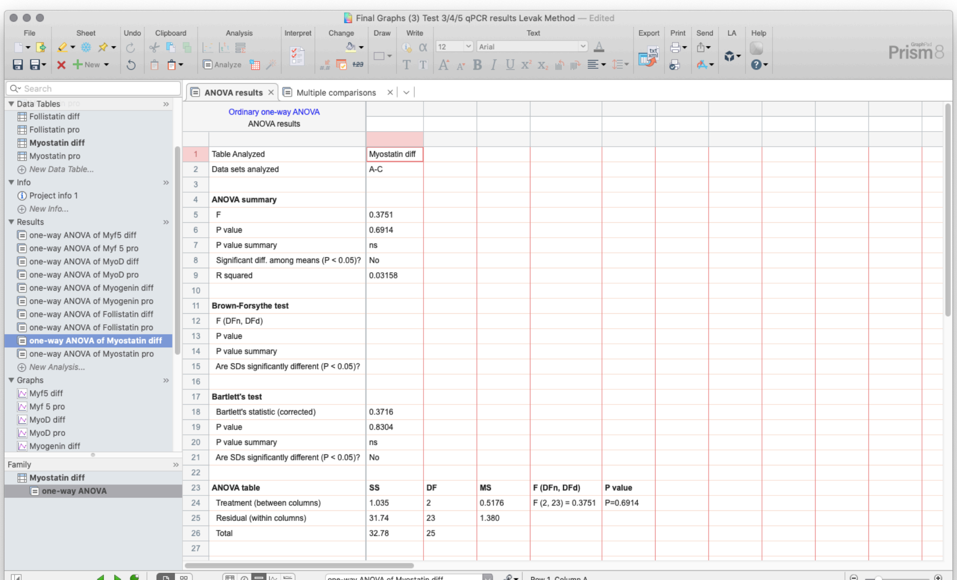

Final Graphs (3) Test 3/4/5 qPCR results Levak Method — Edited
ANOVA results
Multiple comparisons
Ordinary one-way ANOVA
ANOVA results
Table Analyzed | Follistatin pro
Data sets analyzed | A-C
ANOVA summary
F | 0.3831
P value | 0.6882
P value summary | ns
Significant diff. among means (P < 0.05)? | No
R squared | 0.04860
Brown-Forsythe test
F (DFn, DFd)
P value
P value summary
Are SDs significantly different (P < 0.05)?
Bartlett's test
Bartlett's statistic (corrected) | 5.755
P value | 0.0563
P value summary | ns
Are SDs significantly different (P < 0.05)? | No
ANOVA table | SS | DF | MS | F (DFn, DFd) | P value
Treatment (between columns) | 0.1420 | 2 | 0.07100 | F (2, 15) = 0.3831 | P=0.6882
Residual (within columns) | 2.780 | 15 | 0.1853
Total | 2.922 | 17
one-way ANOVA of Follistatin pro
Row 1, Column A



Final Graphs (3) Test 3/4/5 qPCR results Levak Method — Edited

Ordinary one-way ANOVA – Multiple comparisons (one-way ANOVA of Follistatin pro)

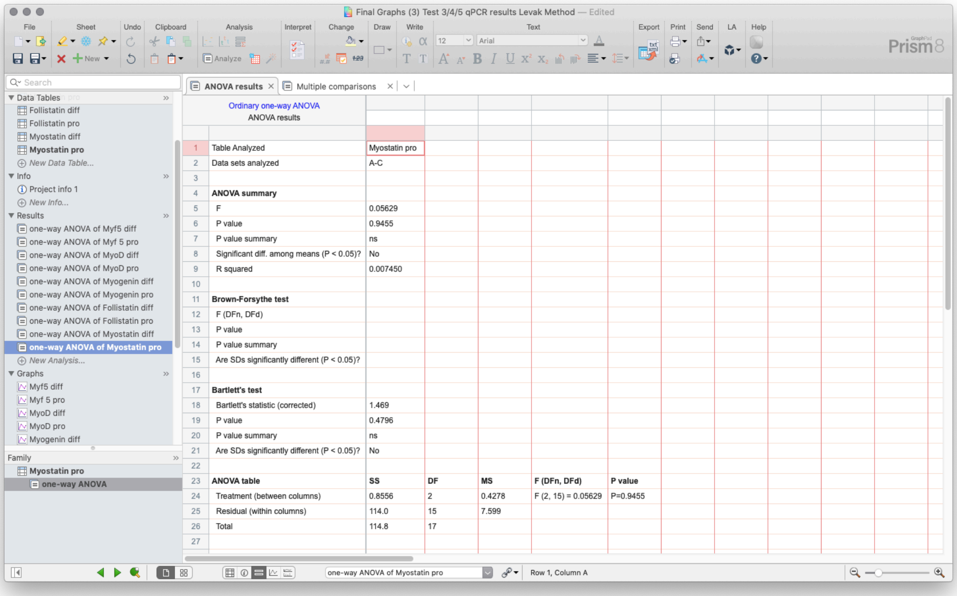
1	Number of families	1							
2	Number of comparisons per family	3							
3	Alpha	0.05							
4									
5	**Tukey's multiple comparisons test**	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**			
6	CBD vs. CBN	0.08891	-0.5567 to 0.7345	No	ns	0.9322	A-B		
7	CBD vs. MTH	-0.1275	-0.7731 to 0.5181	No	ns	0.8662	A-C		
8	CBN vs. MTH	-0.2164	-0.8620 to 0.4292	No	ns	0.6662	B-C		
9									
10	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
11	CBD vs. CBN	0.8725	0.7836	0.08891	0.2485	6	6	0.5059	15
12	CBD vs. MTH	0.8725	1.000	-0.1275	0.2485	6	6	0.7255	15
13	CBN vs. MTH	0.7836	1.000	-0.2164	0.2485	6	6	1.231	15



Final Graphs (3) Test 3/4/5 qPCR results Levak Method — Edited

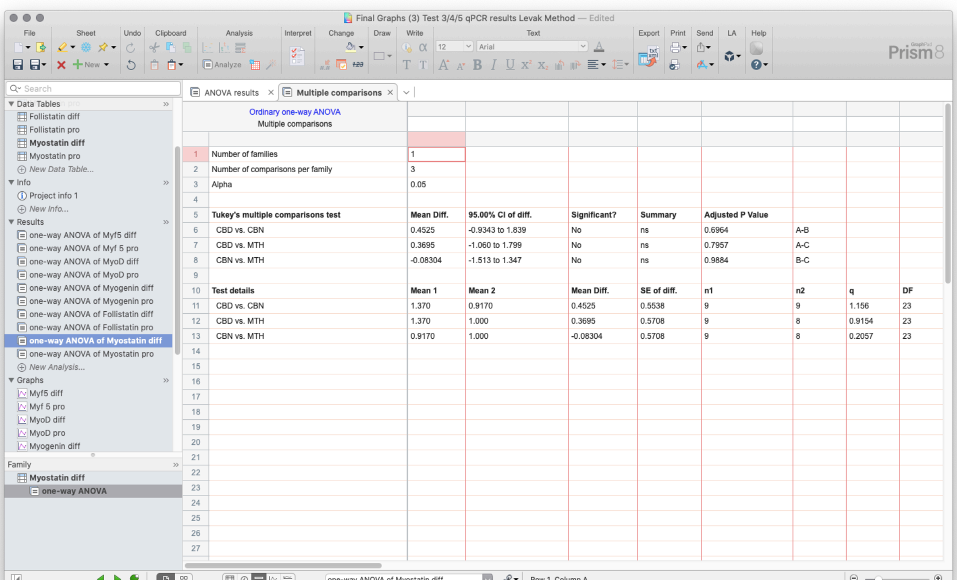
Ordinary one-way ANOVA – ANOVA results (one-way ANOVA of Myostatin diff)

1	Table Analyzed	Myostatin diff				
2	Data sets analyzed	A-C				
3						
4	**ANOVA summary**					
5	F	0.3751				
6	P value	0.6914				
7	P value summary	ns				
8	Significant diff. among means (P < 0.05)?	No				
9	R squared	0.03158				
10						
11	**Brown-Forsythe test**					
12	F (DFn, DFd)					
13	P value					
14	P value summary					
15	Are SDs significantly different (P < 0.05)?					
16						
17	**Bartlett's test**					
18	Bartlett's statistic (corrected)	0.3716				
19	P value	0.8304				
20	P value summary	ns				
21	Are SDs significantly different (P < 0.05)?	No				
22						
23	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
24	Treatment (between columns)	1.035	2	0.5176	F (2, 23) = 0.3751	P=0.6914
25	Residual (within columns)	31.74	23	1.380		
26	Total	32.78	25			



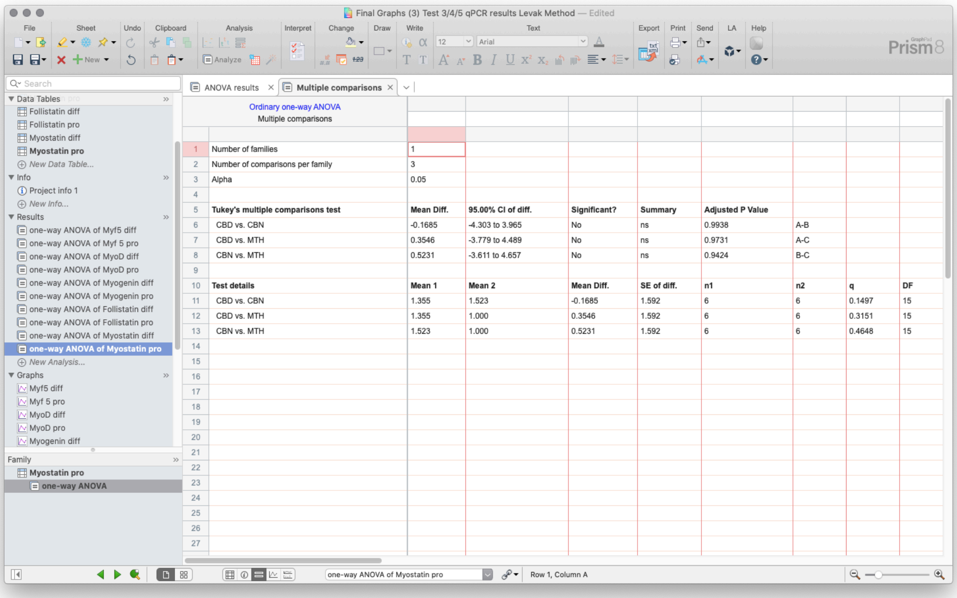
Ordinary one-way ANOVA
ANOVA results

1	Table Analyzed	Myostatin pro				
2	Data sets analyzed	A-C				
3						
4	**ANOVA summary**					
5	F	0.05629				
6	P value	0.9455				
7	P value summary	ns				
8	Significant diff. among means (P < 0.05)?	No				
9	R squared	0.007450				
10						
11	**Brown-Forsythe test**					
12	F (DFn, DFd)					
13	P value					
14	P value summary					
15	Are SDs significantly different (P < 0.05)?					
16						
17	**Bartlett's test**					
18	Bartlett's statistic (corrected)	1.469				
19	P value	0.4796				
20	P value summary	ns				
21	Are SDs significantly different (P < 0.05)?	No				
22						
23	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
24	Treatment (between columns)	0.8556	2	0.4278	F (2, 15) = 0.05629	P=0.9455
25	Residual (within columns)	114.0	15	7.599		
26	Total	114.8	17			



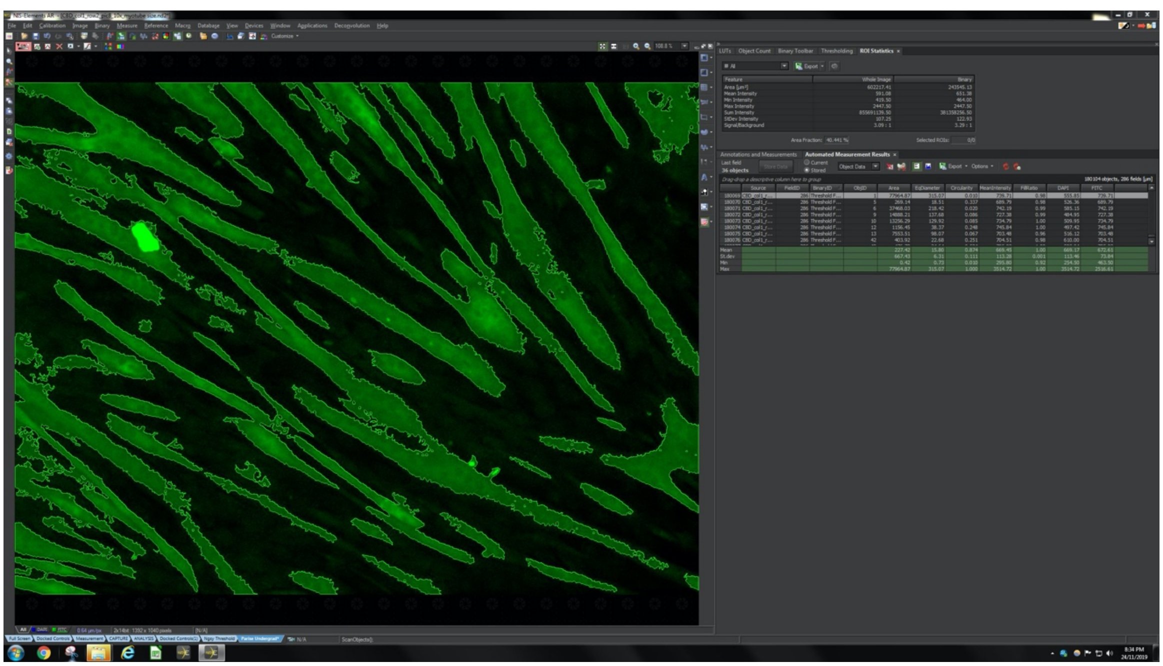
Ordinary one-way ANOVA
Multiple comparisons

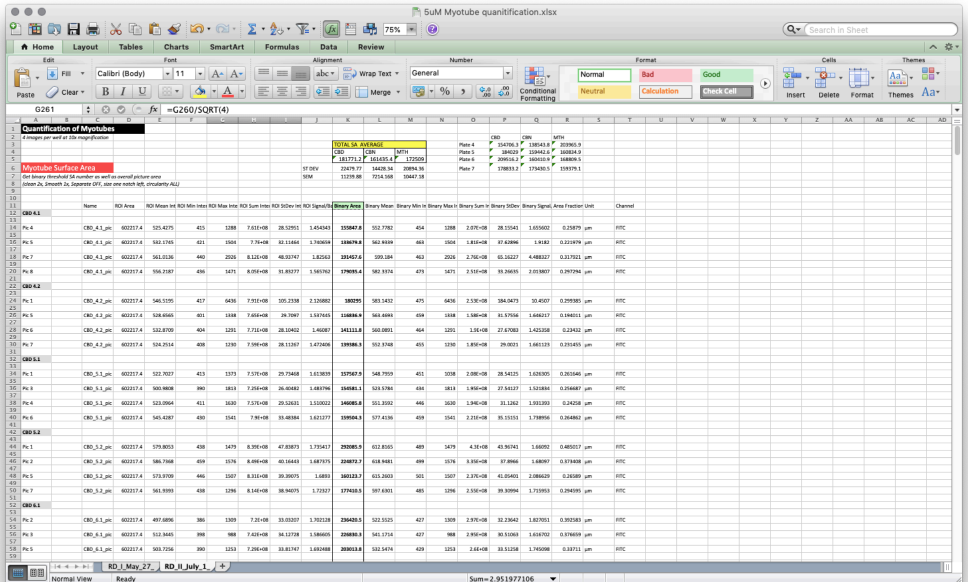
1	Number of families	1							
2	Number of comparisons per family	3							
3	Alpha	0.05							
4									
5	**Tukey's multiple comparisons test**	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**			
6	CBD vs. CBN	0.4525	-0.9343 to 1.839	No	ns	0.6964	A-B		
7	CBD vs. MTH	0.3695	-1.060 to 1.799	No	ns	0.7957	A-C		
8	CBN vs. MTH	-0.08304	-1.513 to 1.347	No	ns	0.9884	B-C		
9									
10	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
11	CBD vs. CBN	1.370	0.9170	0.4525	0.5538	9	9	1.156	23
12	CBD vs. MTH	1.370	1.000	0.3695	0.5708	9	8	0.9154	23
13	CBN vs. MTH	0.9170	1.000	-0.08304	0.5708	9	8	0.2057	23



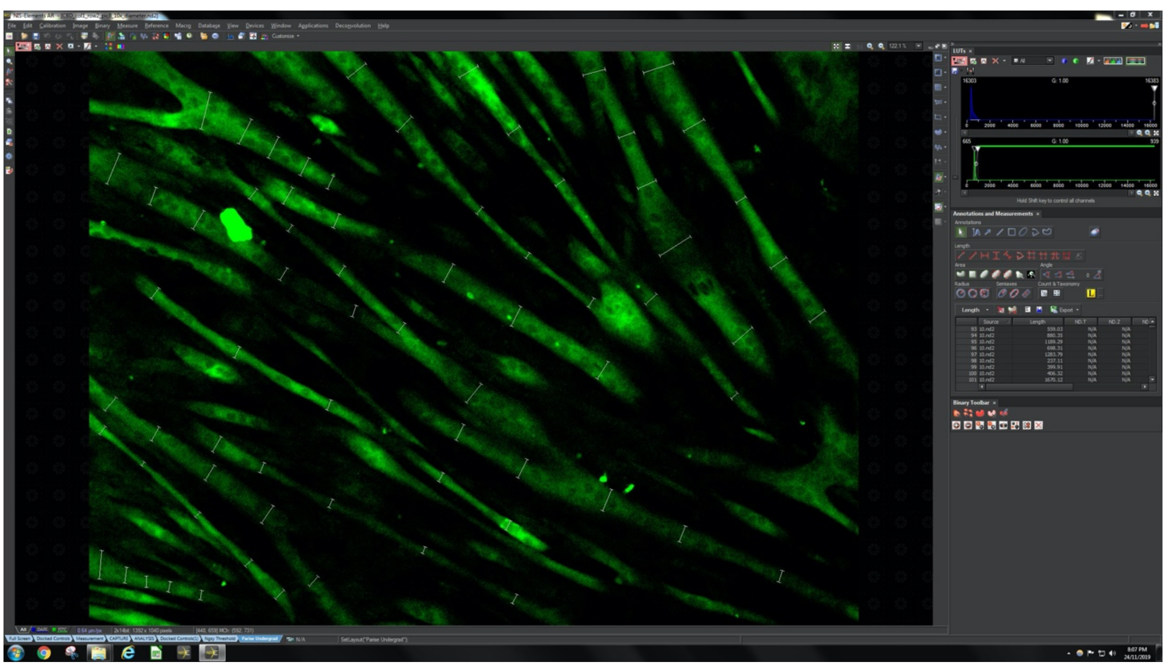
1	Number of families	1							
2	Number of comparisons per family	3							
3	Alpha	0.05							
4									
5	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
6	CBD vs. CBN	-0.1685	-4.303 to 3.965	No	ns	0.9938	A-B		
7	CBD vs. MTH	0.3546	-3.779 to 4.489	No	ns	0.9731	A-C		
8	CBN vs. MTH	0.5231	-3.611 to 4.657	No	ns	0.9424	B-C		
9									
10	Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
11	CBD vs. CBN	1.355	1.523	-0.1685	1.592	6	6	0.1497	15
12	CBD vs. MTH	1.355	1.000	0.3546	1.592	6	6	0.3151	15
13	CBN vs. MTH	1.523	1.000	0.5231	1.592	6	6	0.4648	15

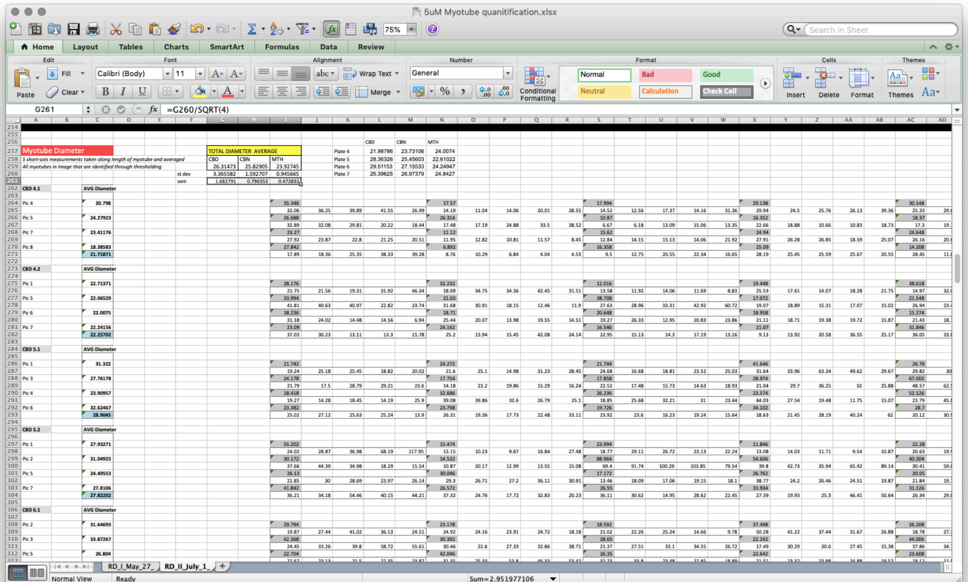
Quantification of Myotube Surface Area (5uM)



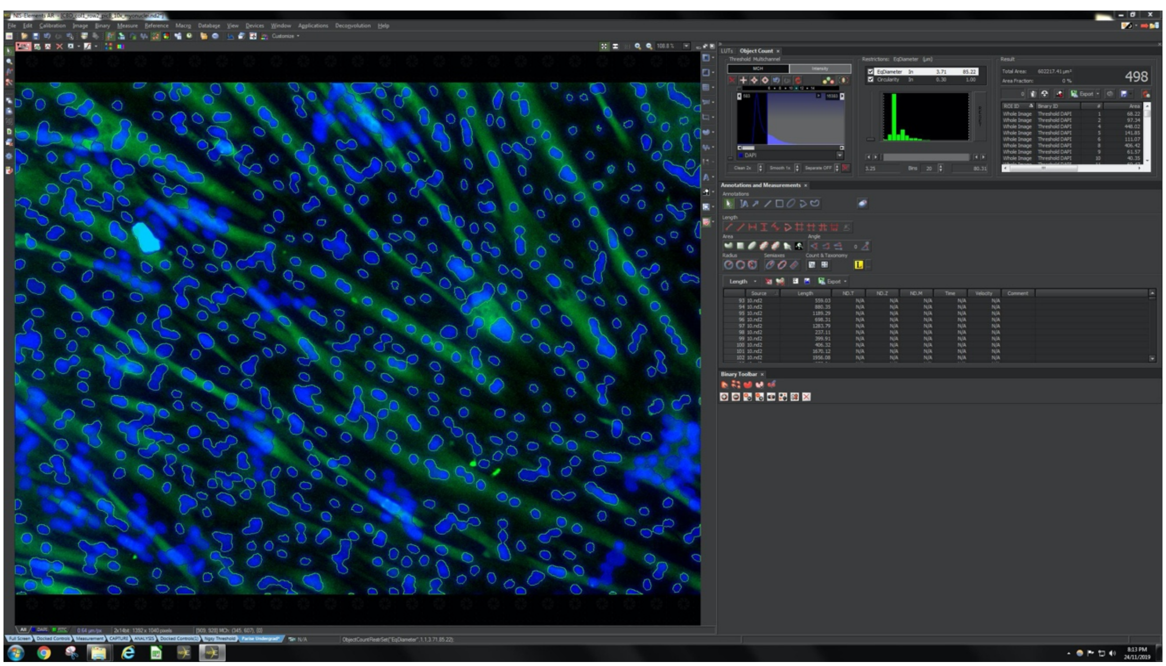


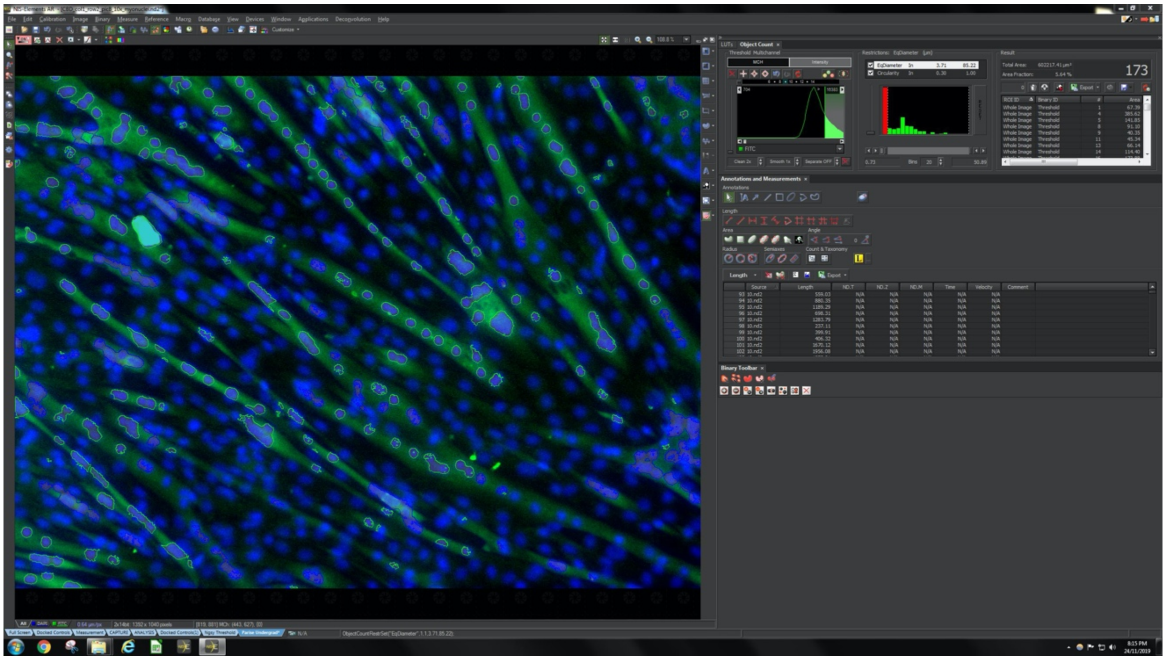
Quantification of Myotube Diameter (5uM)

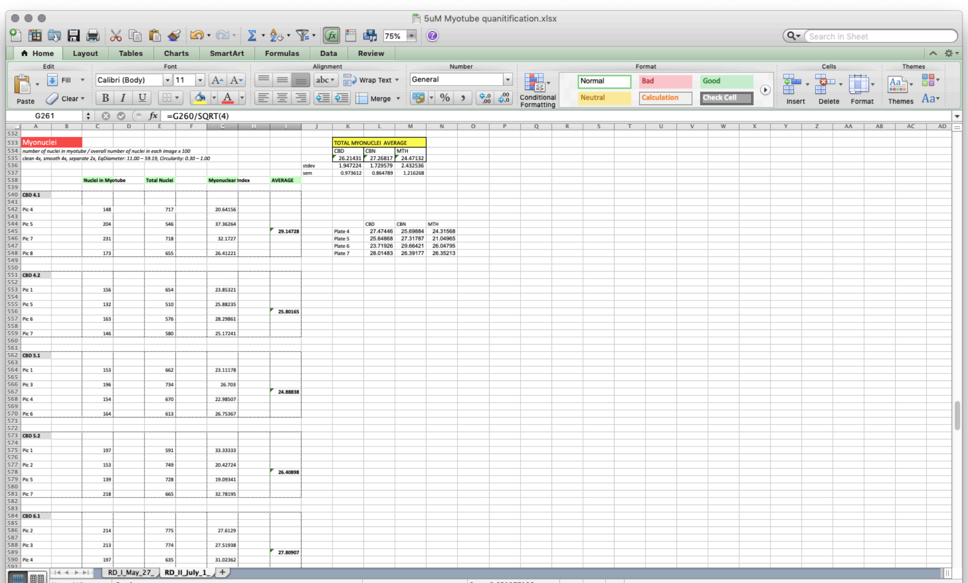




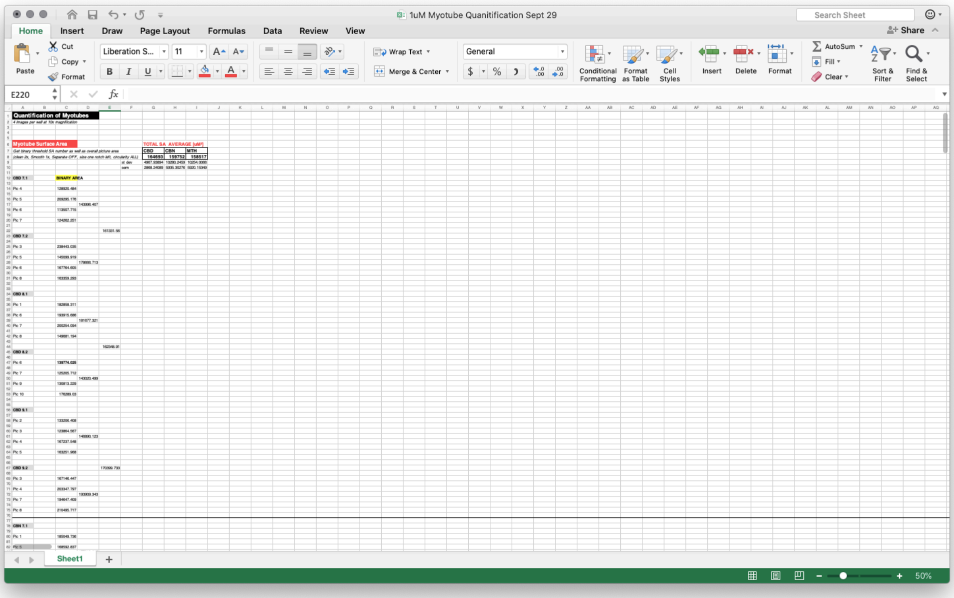
Quantification of Myonuclei (5uM)



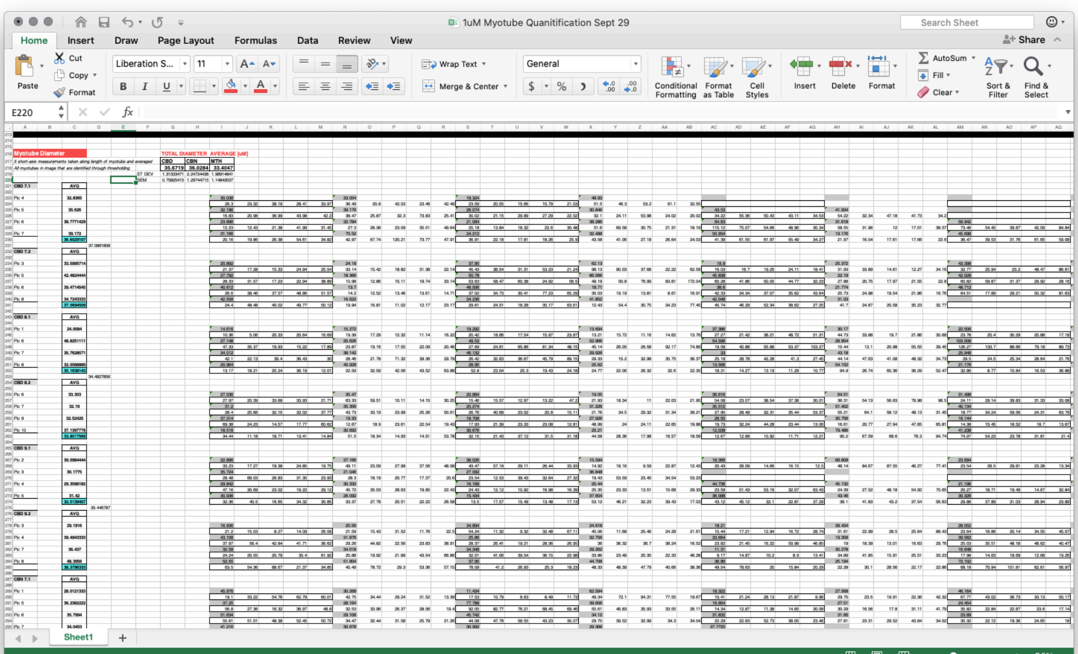




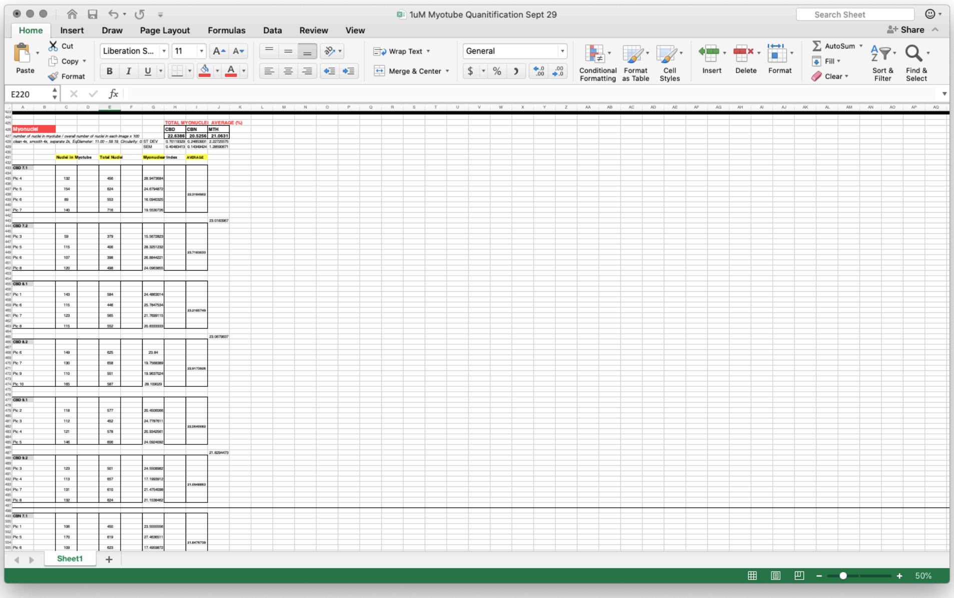
Quantification of Myotube Surface Area (1uM)



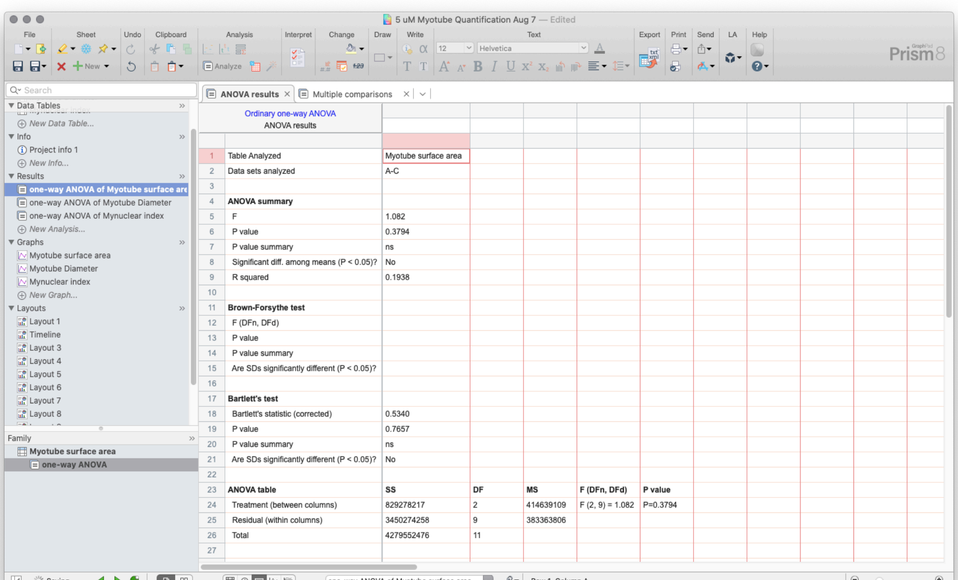
Quantification of Myotube Diameter

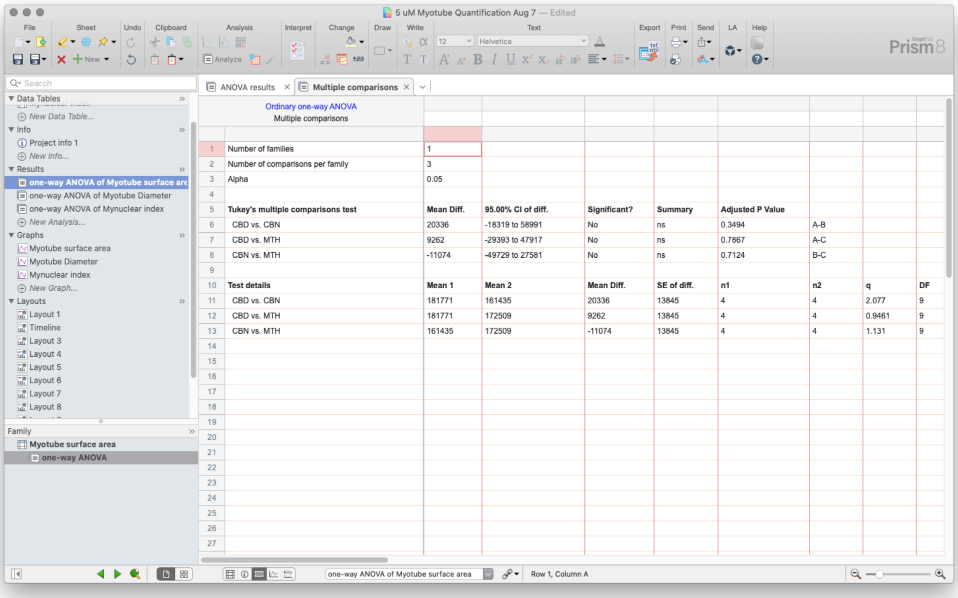


Quantification of Myonuclear Index



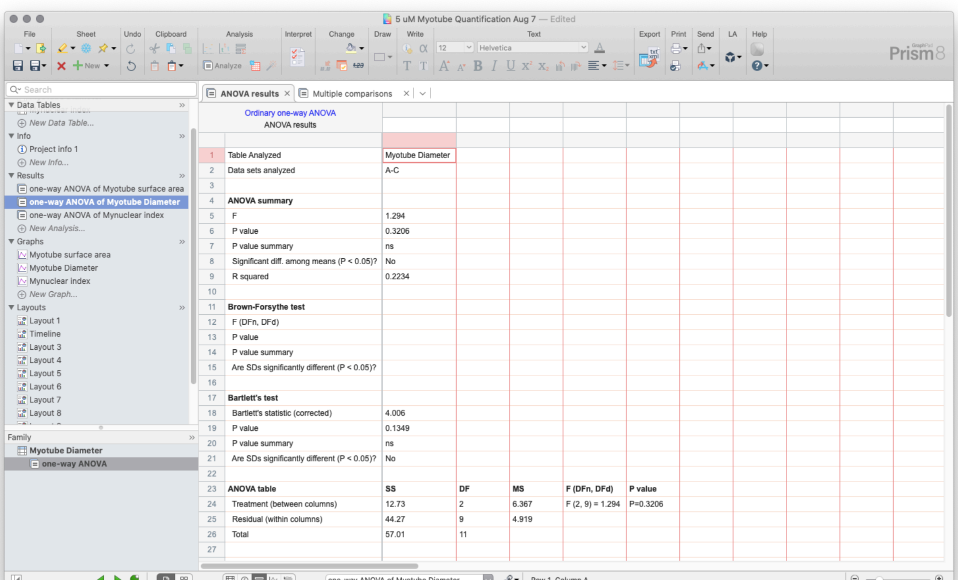
ONE-WAY ANOVA Myotube Formation Analysis on PRISM (5uM)





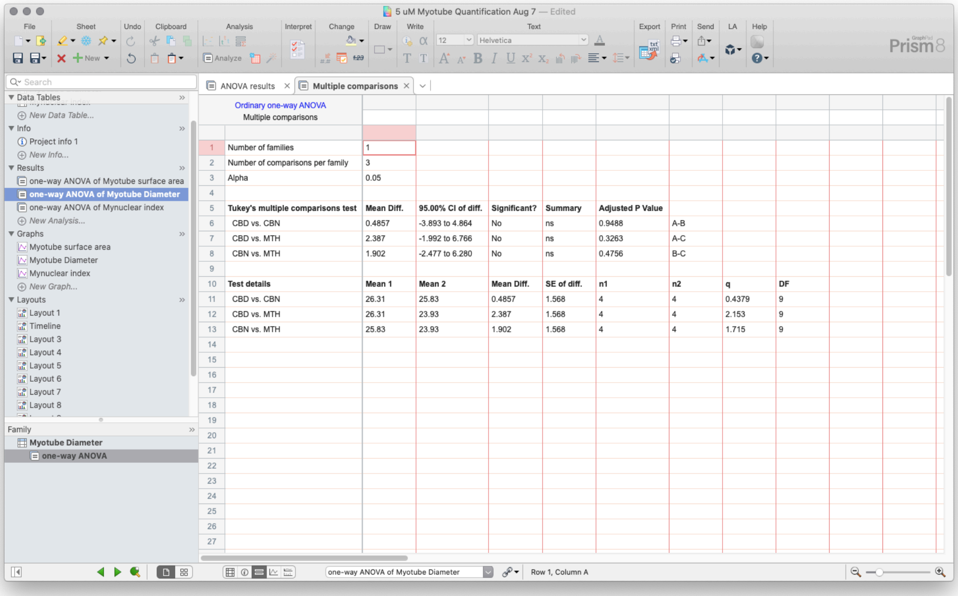
Ordinary one-way ANOVA
Multiple comparisons

1	Number of families	1							
2	Number of comparisons per family	3							
3	Alpha	0.05							
4									
5	**Tukey's multiple comparisons test**	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**			
6	CBD vs. CBN	20336	-18319 to 58991	No	ns	0.3494	A-B		
7	CBD vs. MTH	9262	-29393 to 47917	No	ns	0.7867	A-C		
8	CBN vs. MTH	-11074	-49729 to 27581	No	ns	0.7124	B-C		
9									
10	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
11	CBD vs. CBN	181771	161435	20336	13845	4	4	2.077	9
12	CBD vs. MTH	181771	172509	9262	13845	4	4	0.9461	9
13	CBN vs. MTH	161435	172509	-11074	13845	4	4	1.131	9



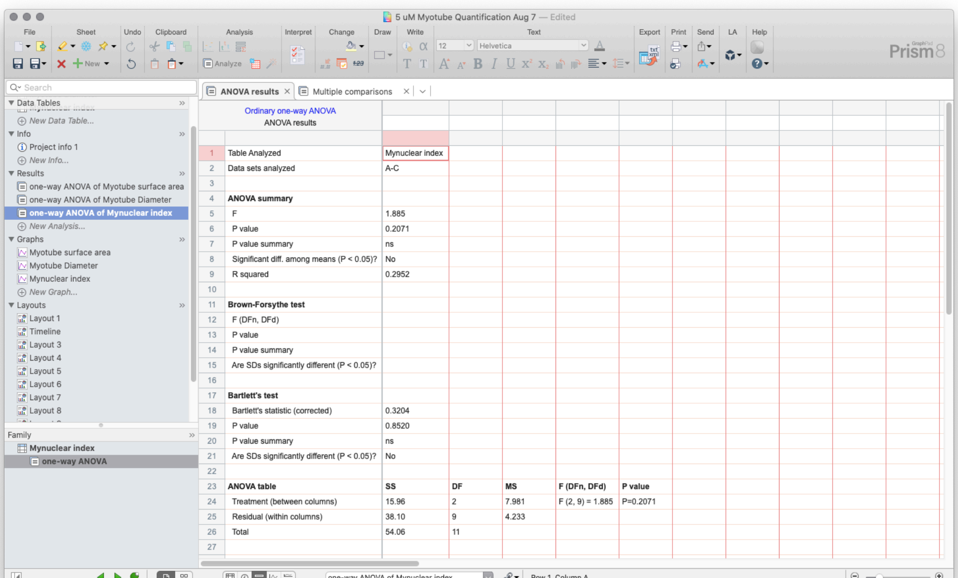
Ordinary one-way ANOVA
ANOVA results

1	Table Analyzed	Myotube Diameter				
2	Data sets analyzed	A-C				
3						
4	**ANOVA summary**					
5	F	1.294				
6	P value	0.3206				
7	P value summary	ns				
8	Significant diff. among means (P < 0.05)?	No				
9	R squared	0.2234				
10						
11	**Brown-Forsythe test**					
12	F (DFn, DFd)					
13	P value					
14	P value summary					
15	Are SDs significantly different (P < 0.05)?					
16						
17	**Bartlett's test**					
18	Bartlett's statistic (corrected)	4.006				
19	P value	0.1349				
20	P value summary	ns				
21	Are SDs significantly different (P < 0.05)?	No				
22						
23	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
24	Treatment (between columns)	12.73	2	6.367	F (2, 9) = 1.294	P=0.3206
25	Residual (within columns)	44.27	9	4.919		
26	Total	57.01	11			



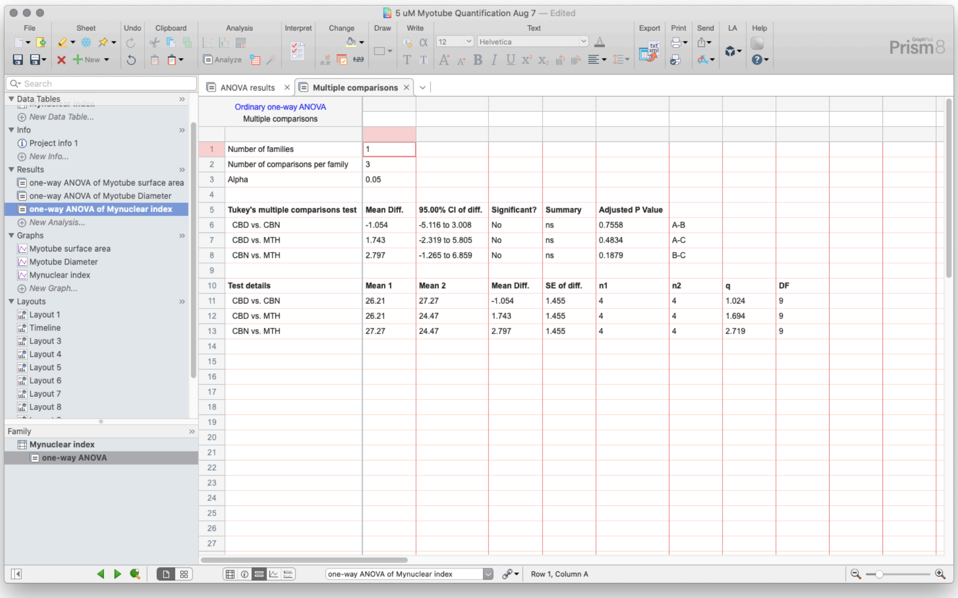
Ordinary one-way ANOVA
Multiple comparisons

1	Number of families	1							
2	Number of comparisons per family	3							
3	Alpha	0.05							
4									
5	**Tukey's multiple comparisons test**	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**			
6	CBD vs. CBN	0.4857	-3.893 to 4.864	No	ns	0.9488	A-B		
7	CBD vs. MTH	2.387	-1.992 to 6.766	No	ns	0.3263	A-C		
8	CBN vs. MTH	1.902	-2.477 to 6.280	No	ns	0.4756	B-C		
9									
10	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
11	CBD vs. CBN	26.31	25.83	0.4857	1.568	4	4	0.4379	9
12	CBD vs. MTH	26.31	23.93	2.387	1.568	4	4	2.153	9
13	CBN vs. MTH	25.83	23.93	1.902	1.568	4	4	1.715	9

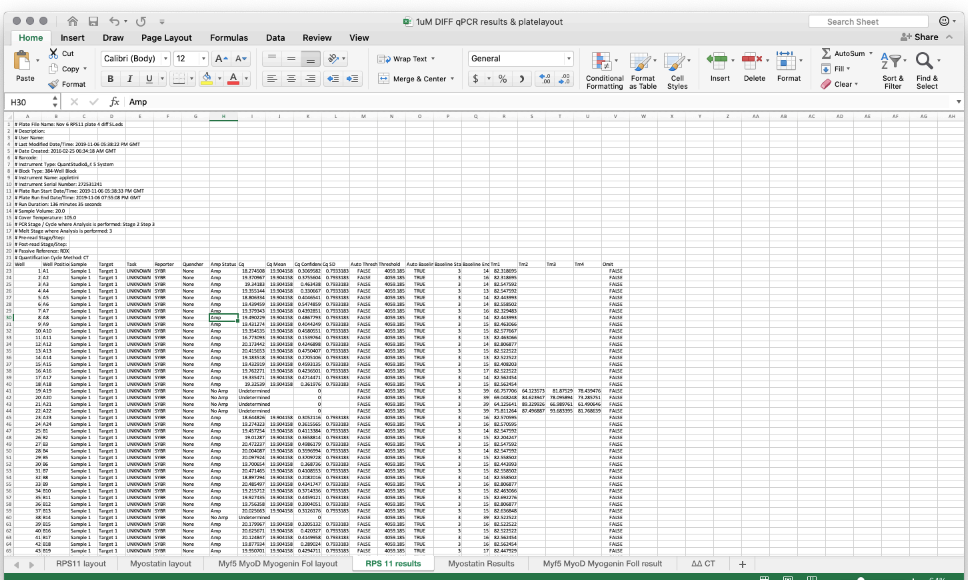


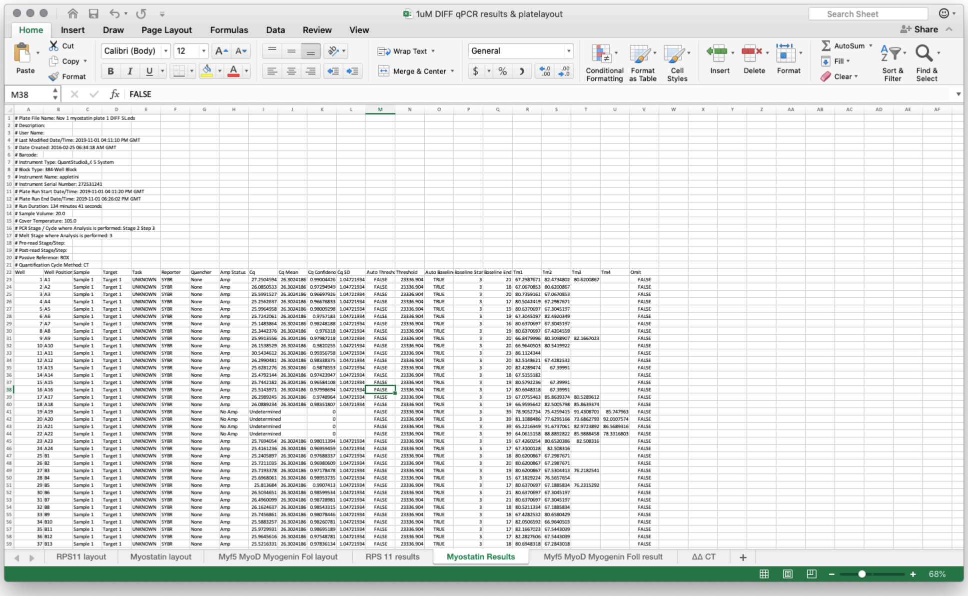
Ordinary one-way ANOVA
ANOVA results

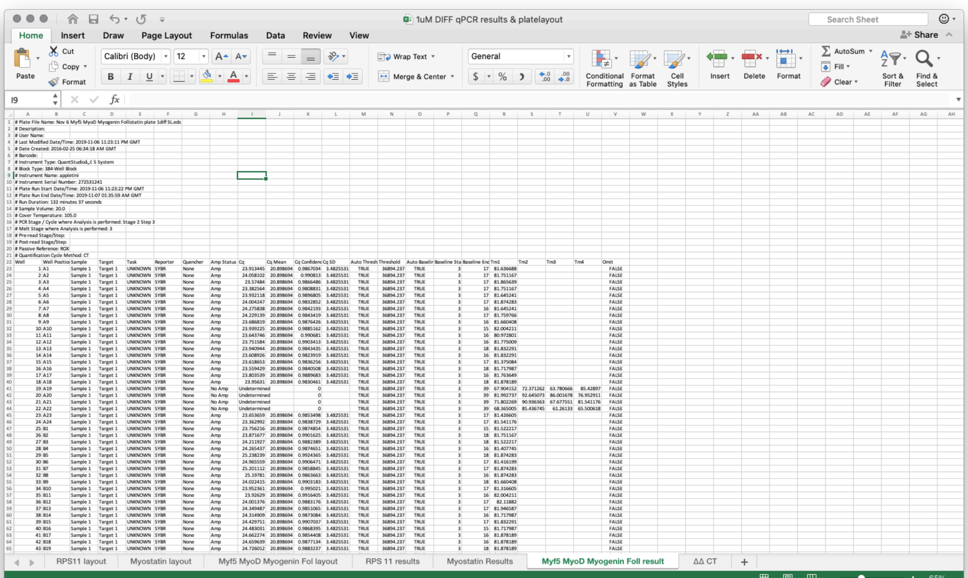
1	Table Analyzed	Mynuclear index				
2	Data sets analyzed	A-C				
3						
4	**ANOVA summary**					
5	F	1.885				
6	P value	0.2071				
7	P value summary	ns				
8	Significant diff. among means (P < 0.05)?	No				
9	R squared	0.2952				
10						
11	**Brown-Forsythe test**					
12	F (DFn, DFd)					
13	P value					
14	P value summary					
15	Are SDs significantly different (P < 0.05)?					
16						
17	**Bartlett's test**					
18	Bartlett's statistic (corrected)	0.3204				
19	P value	0.8520				
20	P value summary	ns				
21	Are SDs significantly different (P < 0.05)?	No				
22						
23	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
24	Treatment (between columns)	15.96	2	7.981	F (2, 9) = 1.885	P=0.2071
25	Residual (within columns)	38.10	9	4.233		
26	Total	54.06	11			

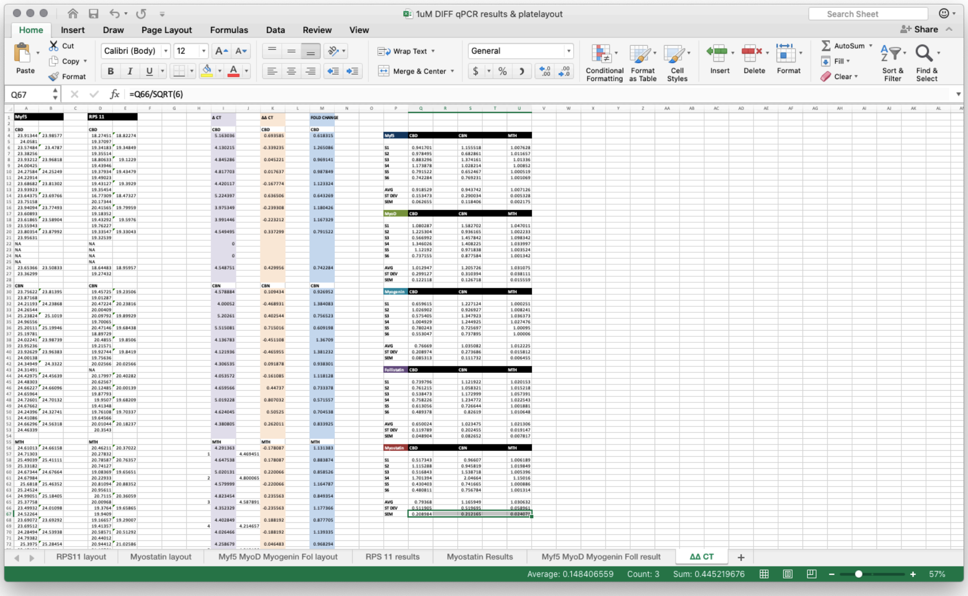


RAW DATA qPCR Results (1uM)

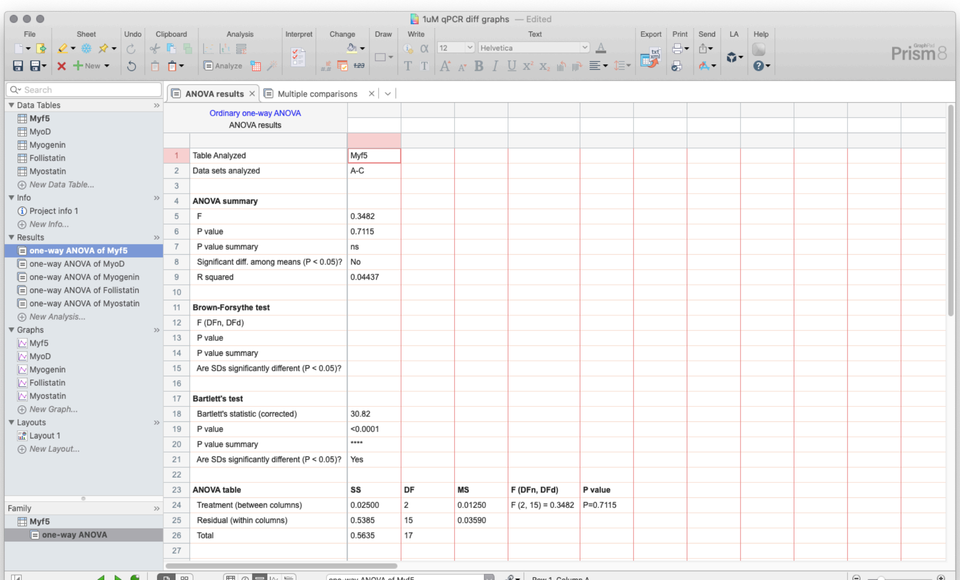


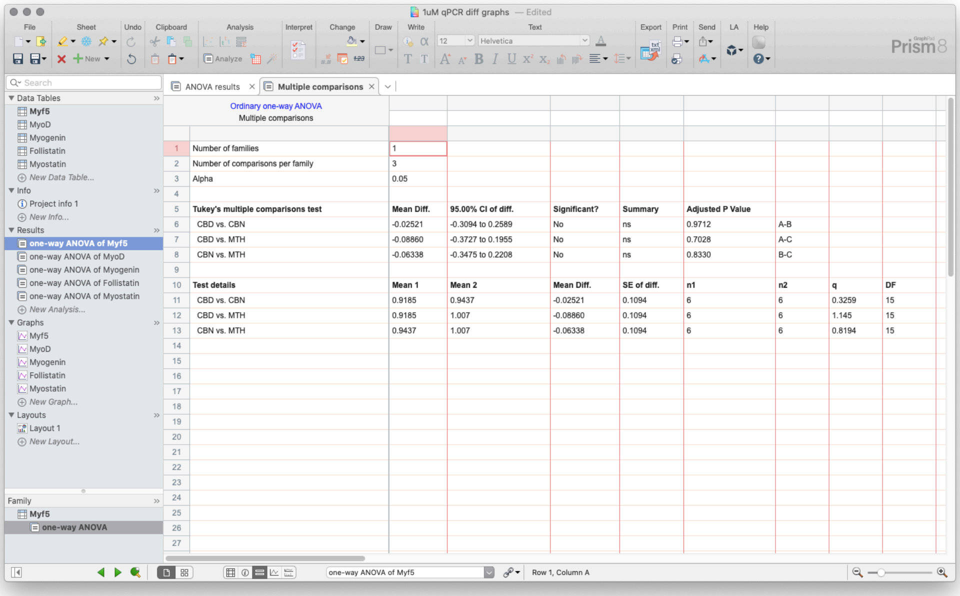

1uM DIFF qPCR results & platelayout
RPS11 layout
Myostatin layout
Myf5 MyoD Myogenin Fol layout
RPS 11 results
Myostatin Results
Myf5 MyoD Myogenin Foll result
ΔΔ CT


1uM DIFF qPCR results & platelayout
RPS11 layout
Myostatin layout
Myf5 MyoD Myogenin Fol layout
RPS 11 results
Myostatin Results
Myf5 MyoD Myogenin Foll result
ΔΔ CT

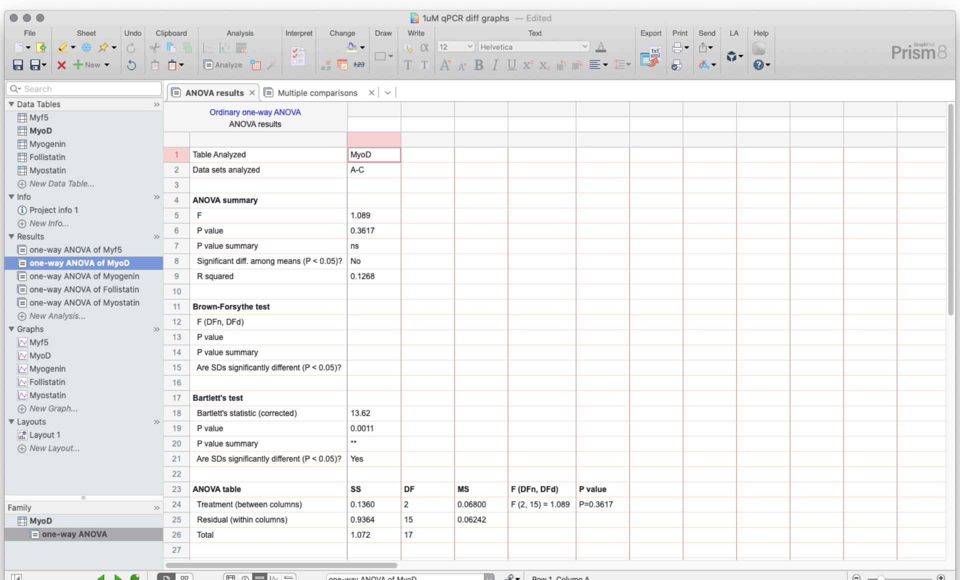


ONE-WAY ANOVA qPCR (1uM)

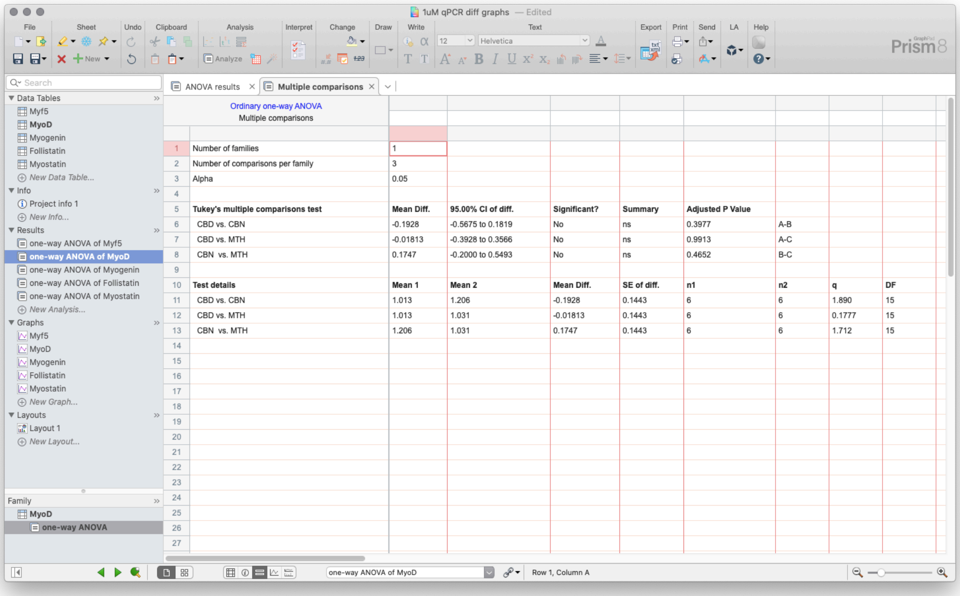




	Ordinary one-way ANOVA Multiple comparisons								
1	Number of families	1							
2	Number of comparisons per family	3							
3	Alpha	0.05							
4									
5	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
6	CBD vs. CBN	-0.02521	-0.3094 to 0.2589	No	ns	0.9712	A-B		
7	CBD vs. MTH	-0.08860	-0.3727 to 0.1955	No	ns	0.7028	A-C		
8	CBN vs. MTH	-0.06338	-0.3475 to 0.2208	No	ns	0.8330	B-C		
9									
10	Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
11	CBD vs. CBN	0.9185	0.9437	-0.02521	0.1094	6	6	0.3259	15
12	CBD vs. MTH	0.9185	1.007	-0.08860	0.1094	6	6	1.145	15
13	CBN vs. MTH	0.9437	1.007	-0.06338	0.1094	6	6	0.8194	15

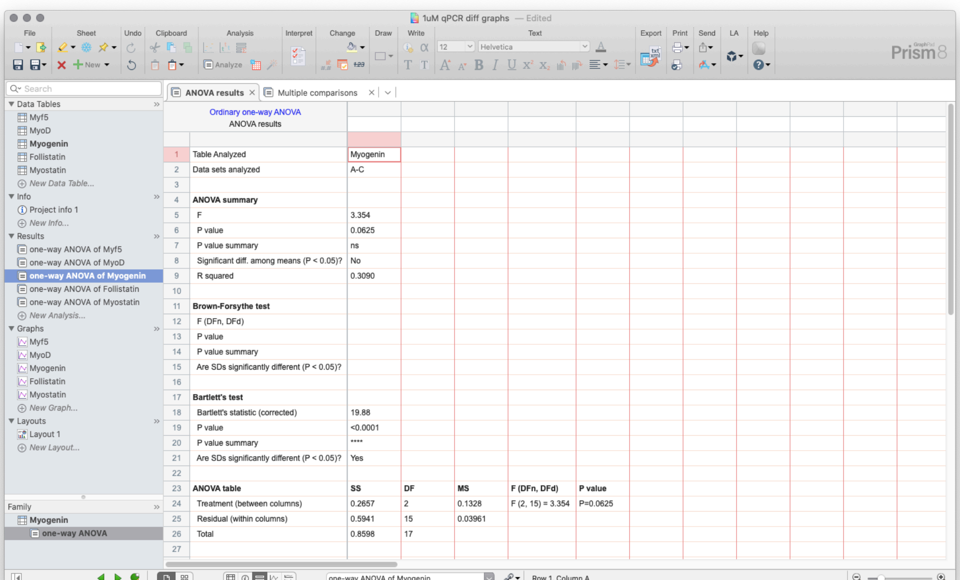


	Ordinary one-way ANOVA ANOVA results					
1	Table Analyzed	MyoD				
2	Data sets analyzed	A-C				
3						
4	ANOVA summary					
5	F	1.089				
6	P value	0.3617				
7	P value summary	ns				
8	Significant diff. among means (P < 0.05)?	No				
9	R squared	0.1268				
10						
11	Brown-Forsythe test					
12	F (DFn, DFd)					
13	P value					
14	P value summary					
15	Are SDs significantly different (P < 0.05)?					
16						
17	Bartlett's test					
18	Bartlett's statistic (corrected)	13.62				
19	P value	0.0011				
20	P value summary	**				
21	Are SDs significantly different (P < 0.05)?	Yes				
22						
23	ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
24	Treatment (between columns)	0.1360	2	0.06800	F (2, 15) = 1.089	P=0.3617
25	Residual (within columns)	0.9364	15	0.06242		
26	Total	1.072	17			



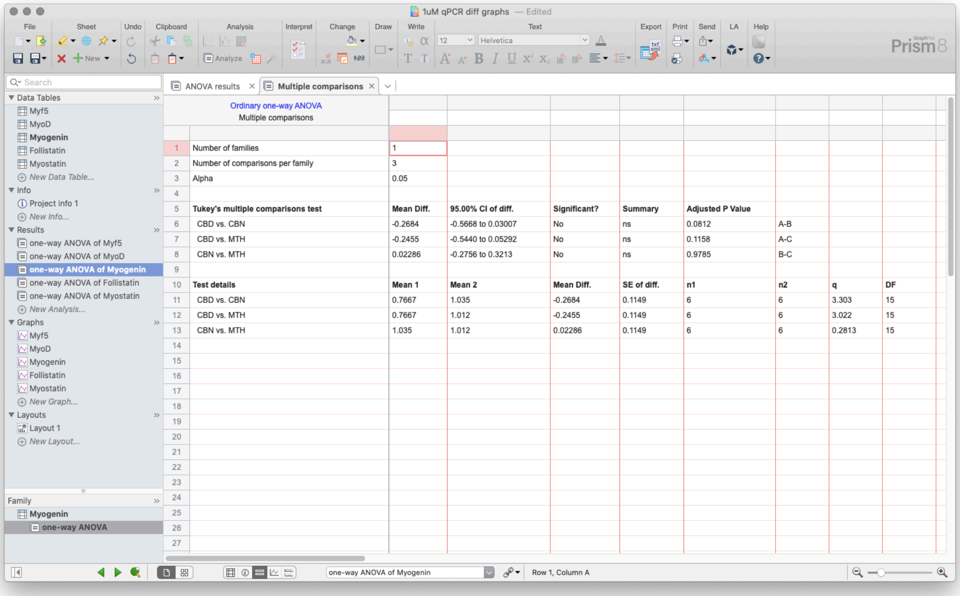
Ordinary one-way ANOVA
Multiple comparisons

1	Number of families	1							
2	Number of comparisons per family	3							
3	Alpha	0.05							
4									
5	**Tukey's multiple comparisons test**	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**			
6	CBD vs. CBN	-0.1928	-0.5675 to 0.1819	No	ns	0.3977	A-B		
7	CBD vs. MTH	-0.01813	-0.3928 to 0.3566	No	ns	0.9913	A-C		
8	CBN vs. MTH	0.1747	-0.2000 to 0.5493	No	ns	0.4652	B-C		
9									
10	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
11	CBD vs. CBN	1.013	1.206	-0.1928	0.1443	6	6	1.890	15
12	CBD vs. MTH	1.013	1.031	-0.01813	0.1443	6	6	0.1777	15
13	CBN vs. MTH	1.206	1.031	0.1747	0.1443	6	6	1.712	15



Ordinary one-way ANOVA
ANOVA results

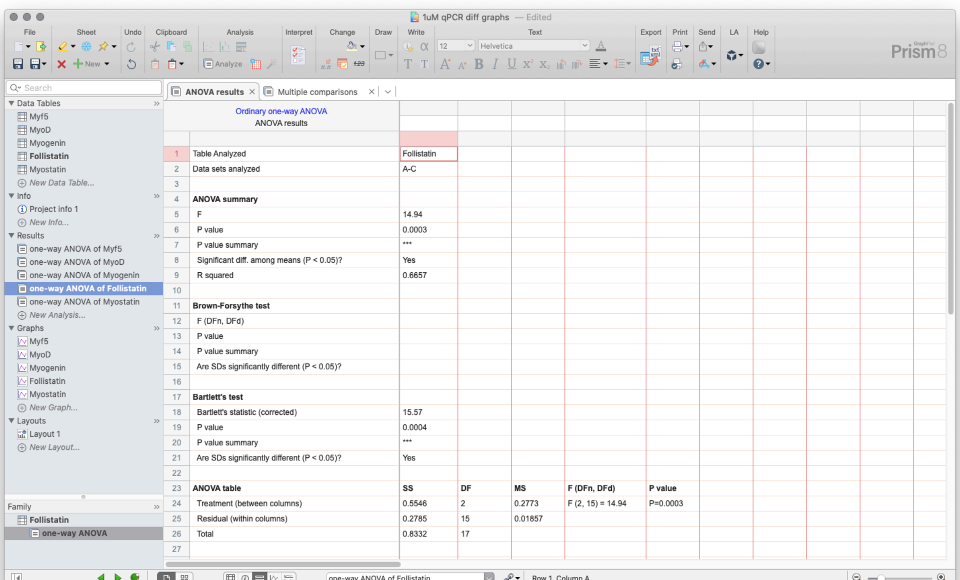
1	Table Analyzed	Myogenin				
2	Data sets analyzed	A-C				
3						
4	**ANOVA summary**					
5	F	3.354				
6	P value	0.0625				
7	P value summary	ns				
8	Significant diff. among means (P < 0.05)?	No				
9	R squared	0.3090				
10						
11	**Brown-Forsythe test**					
12	F (DFn, DFd)					
13	P value					
14	P value summary					
15	Are SDs significantly different (P < 0.05)?					
16						
17	**Bartlett's test**					
18	Bartlett's statistic (corrected)	19.88				
19	P value	<0.0001				
20	P value summary	****				
21	Are SDs significantly different (P < 0.05)?	Yes				
22						
23	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
24	Treatment (between columns)	0.2657	2	0.1328	F (2, 15) = 3.354	P=0.0625
25	Residual (within columns)	0.5941	15	0.03961		
26	Total	0.8598	17			



1uM qPCR diff graphs — Edited

Ordinary one-way ANOVA
Multiple comparisons

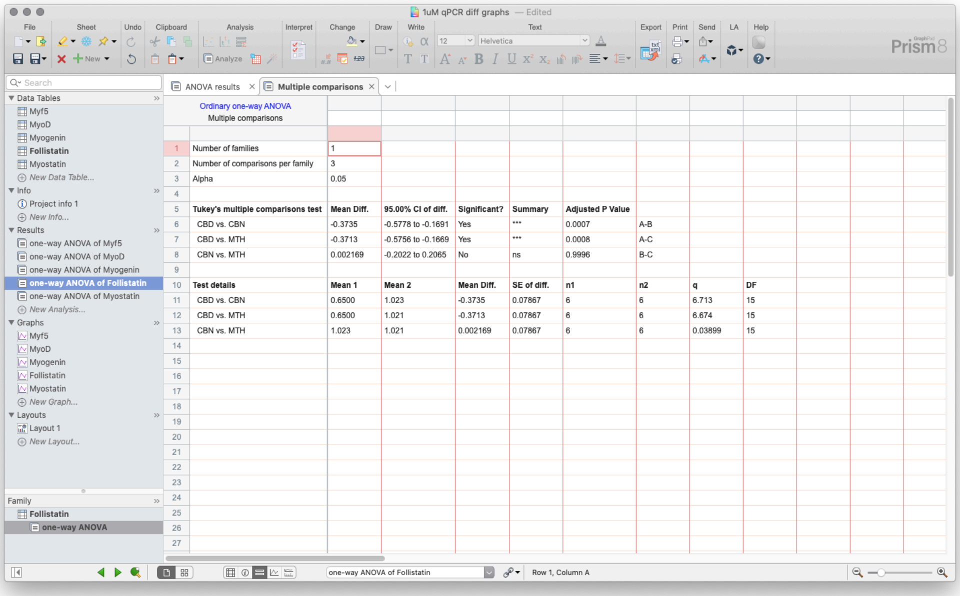
1	Number of families	1							
2	Number of comparisons per family	3							
3	Alpha	0.05							
4									
5	**Tukey's multiple comparisons test**	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**			
6	CBD vs. CBN	-0.2684	-0.5668 to 0.03007	No	ns	0.0812	A-B		
7	CBD vs. MTH	-0.2455	-0.5440 to 0.05292	No	ns	0.1158	A-C		
8	CBN vs. MTH	0.02286	-0.2756 to 0.3213	No	ns	0.9785	B-C		
9									
10	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
11	CBD vs. CBN	0.7667	1.035	-0.2684	0.1149	6	6	3.303	15
12	CBD vs. MTH	0.7667	1.012	-0.2455	0.1149	6	6	3.022	15
13	CBN vs. MTH	1.035	1.012	0.02286	0.1149	6	6	0.2813	15



1uM qPCR diff graphs — Edited

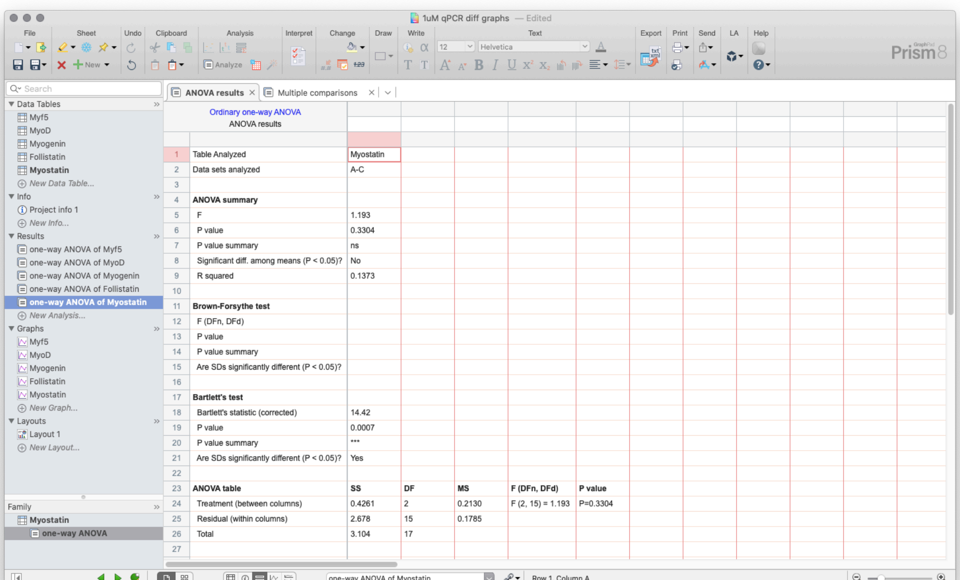
Ordinary one-way ANOVA
ANOVA results

1	Table Analyzed	Follistatin				
2	Data sets analyzed	A-C				
3						
4	**ANOVA summary**					
5	F	14.94				
6	P value	0.0003				
7	P value summary	***				
8	Significant diff. among means (P < 0.05)?	Yes				
9	R squared	0.6657				
10						
11	**Brown-Forsythe test**					
12	F (DFn, DFd)					
13	P value					
14	P value summary					
15	Are SDs significantly different (P < 0.05)?					
16						
17	**Bartlett's test**					
18	Bartlett's statistic (corrected)	15.57				
19	P value	0.0004				
20	P value summary	***				
21	Are SDs significantly different (P < 0.05)?	Yes				
22						
23	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
24	Treatment (between columns)	0.5546	2	0.2773	F (2, 15) = 14.94	P=0.0003
25	Residual (within columns)	0.2785	15	0.01857		
26	Total	0.8332	17			



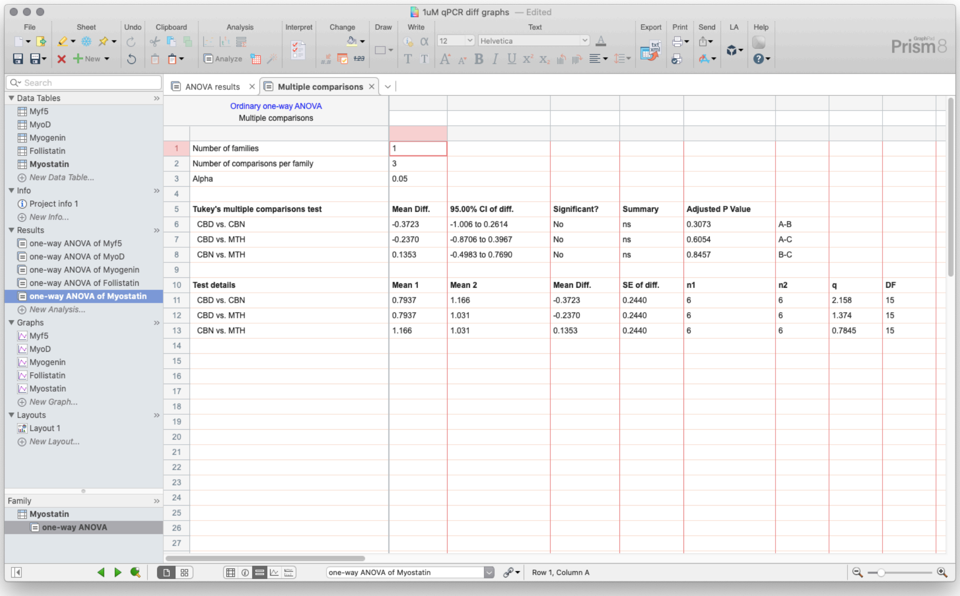
Ordinary one-way ANOVA
Multiple comparisons

1	Number of families	1							
2	Number of comparisons per family	3							
3	Alpha	0.05							
4									
5	**Tukey's multiple comparisons test**	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**			
6	CBD vs. CBN	-0.3735	-0.5778 to -0.1691	Yes	***	0.0007	A-B		
7	CBD vs. MTH	-0.3713	-0.5756 to -0.1669	Yes	***	0.0008	A-C		
8	CBN vs. MTH	0.002169	-0.2022 to 0.2065	No	ns	0.9996	B-C		
9									
10	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
11	CBD vs. CBN	0.6500	1.023	-0.3735	0.07867	6	6	6.713	15
12	CBD vs. MTH	0.6500	1.021	-0.3713	0.07867	6	6	6.674	15
13	CBN vs. MTH	1.023	1.021	0.002169	0.07867	6	6	0.03899	15



Ordinary one-way ANOVA
ANOVA results

1	Table Analyzed	Myostatin				
2	Data sets analyzed	A-C				
3						
4	**ANOVA summary**					
5	F	1.193				
6	P value	0.3304				
7	P value summary	ns				
8	Significant diff. among means (P < 0.05)?	No				
9	R squared	0.1373				
10						
11	**Brown-Forsythe test**					
12	F (DFn, DFd)					
13	P value					
14	P value summary					
15	Are SDs significantly different (P < 0.05)?					
16						
17	**Bartlett's test**					
18	Bartlett's statistic (corrected)	14.42				
19	P value	0.0007				
20	P value summary	***				
21	Are SDs significantly different (P < 0.05)?	Yes				
22						
23	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
24	Treatment (between columns)	0.4261	2	0.2130	F (2, 15) = 1.193	P=0.3304
25	Residual (within columns)	2.678	15	0.1785		
26	Total	3.104	17			



Ordinary one-way ANOVA
Multiple comparisons

1	Number of families	1							
2	Number of comparisons per family	3							
3	Alpha	0.05							
4									
5	**Tukey's multiple comparisons test**	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**			
6	CBD vs. CBN	-0.3723	-1.006 to 0.2614	No	ns	0.3073	A-B		
7	CBD vs. MTH	-0.2370	-0.8706 to 0.3967	No	ns	0.6054	A-C		
8	CBN vs. MTH	0.1353	-0.4983 to 0.7690	No	ns	0.8457	B-C		
9									
10	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
11	CBD vs. CBN	0.7937	1.166	-0.3723	0.2440	6	6	2.158	15
12	CBD vs. MTH	0.7937	1.031	-0.2370	0.2440	6	6	1.374	15
13	CBN vs. MTH	1.166	1.031	0.1353	0.2440	6	6	0.7845	15

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