The Effect of Interleukin 10 and Peptide derivatives on Macrophages Involved in Liver Fibrosis

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Abstract

Liver fibrosis is a disease characterized by liver injury and replacement of injured tissue with collagenous scar. IL-10 and its peptide derivatives are potent molecules in treatment of liver fibrosis. IL-10 and the peptides contain inflammatory properties which can be useful in treatment of liver fibrosis. In this study, by finding an optimal method, the inflammatory effects of IL-10 and peptide derivatives were assessed on RAW 264.7 macrophages. This was done by performing several NO assays under different conditions to determine the NO concentrations as well as performing qPCR to determine relative TNF-α expression levels after treatment with IL-10 and the peptides. IL-10 showed some, but very little reduction in NO concentration after treatment, whereas the peptides often caused an increase in NO concentration. IL-10 was found to greatly reduce relative TNF-α mRNA expression. The peptides caused an increase in relative TNF-α mRNA expression. The results indicated that it is not possible to conclude anti-inflammatory effects of IL-10 on NO production, but it does significantly decrease relative TNF-α mRNA expression levels. The peptides did not display any anti-inflammatory activity, rather they showed pro-inflammatory effects. It is suggested to perform further research on this and the anti-fibrotic effects of the peptides.

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Introduction

Liver fibrosis is a widely known disease with an estimated number of cases to be 1.5 billion worldwide [1]. It is characterized by liver injury in which the injured tissue is replaced by a collagenous scar. Liver fibrosis can develop further at different rates depending on several factors. If this injury and inflammation continue to progress, liver cirrhosis can occur. Liver cirrhosis is an advanced stage of liver fibrosis in which there is also a distortion of the hepatic vasculature present [2].

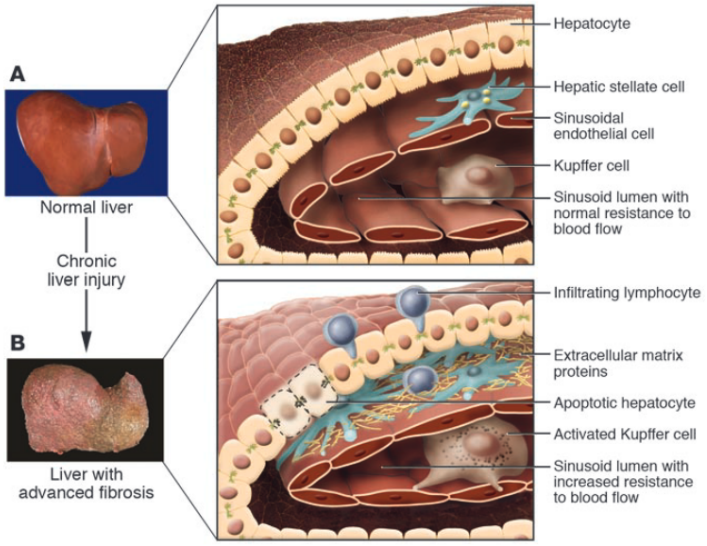

Figure 1 A comparison of cellular composition between a normal liver (A) and a fibrotic liver (B) [4].

The liver consists of cells that are part of the hepatic parenchyma. Cells that are present in this parenchyma are the hepatocytes and the endothelial cells. The hepatic stellate cells and the Kupffer cells are part of the nonparenchymal cells. The hepatic macrophages are part of the nonparenchymal cells and mainly consist of the Kupffer cells [3]. During liver fibrosis, there is more production of extracellular matrix in relation to the degradation of the extracellular matrix in the space of Disse. This results in a decreased functioning of blood supply coming from the hepatic portal vein and traveling to the parenchymal cells [5].
One fundamental change occurring inside the liver due to liver fibrosis is the extracellular matrix consisting of collagen types I and III, whereas a healthy liver mainly consists of collagen types IV and VI [5].

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria. LPS is known to cause immune responses in our body. LPS consists of three major components, namely; lipid A, the core oligosaccharide chain and O-specific chain [6].
Upon exposure of macrophages to LPS, they are polarized into M1 macrophages and inflammation occurs [7]. This is caused by the binding of the lipid A chain of LPS to TLR4 [6]. In order for this binding to successfully happen, LPS needs the LPS binding protein (LBP) as well as CD14, which is responsible for the internalization of LPS-activated TLR4. This binding then activates TLR4, which in turn triggers two signaling cascades; the MyD88-dependent signaling pathway and TRIF-dependent signaling pathway, with the latter activating MAPK [7].

The MyD88-dependent signaling pathway is mainly responsible for the expression of various pro-inflammatory cytokines, like Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and type III interferons (IFN-γ 1/2), by activation of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kβ). The release of these cytokines in the end leads to increased expression of the major histocompatibility complex-II (MHC-II) [7].

Nitric oxide (NO) is a free radical that has a short half-life. It is an internal messenger responsible for mediating functions such as vascular homeostasis, neurotransmission and host defense. Nitric oxide is synthesized from L-arginine by nitric-oxide synthase (NOS). Until today, three different forms of NOS are known, which are endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). In the M1 macrophages, NO is produced by iNOS. If excess NO is produced from iNOS, inflammation can occur [8].

The MyD88-dependent signaling pathway is also known to contribute in the production of IL-10. IL-10 has anti-inflammatory properties to aid in the termination of the inflammation, because IL-10 is known to reduce the activation of NF-kβ, inhibit the expression of TNF-α, and prevent LPS-induced MyD88 augmentation [9]. The uptake of IL-10 by macrophages causes the polarization of M0 or M1 into M2 macrophages. These types are the anti-inflammatory macrophages. IL-10 is effective after its binding to the IL-10 receptor (IL-10R) which is composed of two chains (IL-10R1 and IL-10R2). These receptors are present in the macrophages of the liver as well as other cells of the immune system. After binding to its receptor, IL-10 induces signaling via the transcription factor Signal Transducer and Activator of Transcription 3 (STAT3) [10]. This then results in production of SOCS1 and SOCS3. SOCS3 is known to reduce iNOS production which is responsible for the production of NO. Nitric oxide is one the major components that indicates inflammation. By reducing this NO production, IL-10 also exerts its anti-inflammatory effect [7,8,9,10].

Prof. Dr. K. Poelstra has been able to synthesize three peptides mimicking IL-10 activity, namely P1, P2 and P3. Peptide 1 is the most identical to IL-10. Peptide 2 and 3 are components of this peptide 1, either containing a helix in its structure or not containing the helix. All peptides are potentially pro-inflammatory, while mimicking the anti-fibrotic effects of IL-10. This makes them potent peptides in the treatment of liver fibrosis.

In this study, the effect of IL-10 and three peptide derivatives in the treatment of liver fibrosis is studied. The inflammatory effects of IL-10 and these peptides were determined on the RAW 264.7 macrophages involved in inflammation in the liver. This was done by finding an optimal method to assess these inflammatory properties. These effects were then assessed by determining the nitric oxide production under different conditions. The relative mRNA expression of TNF-alpha under different conditions was also determined by performing a qPCR.

Materials and Methods

The Cell Line

The cells that were used cells for all experiments were RAW 264.7 macrophages (TIB-71), with passage number 8 (p8), isolated from the liver. They were cultured in Dulbecco's Modified Eagle Medium (DMEM, CAT: 32430-027) with 10% fetal boval serum (FBS, heat inactivated) at 37°C and 5% CO_2.

Cytokines and stimulants:

Recombinant murine IFN-γ (CAT: 315-05), recombinant murine IL-10 (CAT: 210-10), Lipopolysaccharides from Escherichia coli (Merck, CAT: L4391), IL-10R1-binding peptide named P1 and three parts of IL-10R1-binding peptide named P2, P3 and P4 (created by Prof. Dr. K. Poelstra, synthesized by Nunzianna Doti, Institute of Biostructures and Bioimaging, Napels, Italy). All cytokines and stimulants were obtained from PeproTech™.

Determination of NO concentration in stimulated RAW 264.7 macrophages

To determine the NO concentration after the treatment of the RAW 264.7 macrophages with cytokines, the RAW 264.7 macrophages ($10*10^4$ cells/well) were first seeded in a 96-well plate for 24 hours at 37°C to make sure that they are settled to the bottom of the well plate. Then, the respective cytokines were added to the wells in triplo. This was incubated for 2 or 24 hours, depending on the type of experiment, and finally the NO concentration was determined by performing an NO assay according to the NO assay protocol.
Appendix C: NO assay protocol

1.1 The effect of LPS and IFN-γ on NO production by RAW 264.7 macrophages.
After 24 hours of incubation at 37°C, 20 µl of 100 ng/ml LPS or 20 µl of 20 ng/ml IFN-γ was added to the cells that were treated with LPS or IFN-γ alone. RAW 264.7 macrophages exposed to co-stimulation of both 100 ng/ml LPS and 20 ng/ml IFN-γ were treated with 20 µl of a mixture of LPS and IFN-γ. After these cytokines were added, the RAW 264.7 macrophages were incubated again at 37°C for 24 hours, after which the NO assay was performed.

1.2 The effect of IL-10 and peptides on production of NO by RAW 264.7 macrophages.
The RAW 264.7 macrophages were stimulated with 100 ng/ml LPS and 20 ng/ml IFN-γ by adding 20 µl of a mixture of both LPS and IFN-γ to all wells except for the control well. This was then incubated at 37°C for 24 hours, after which a sample was taken and stored in a -20°C freezer. Then, 20 µl of either 30 ng/ml IL-10, 30 ng/ml P1 or 30 ng/ml P3 was added for two hours after which another sample was taken and frozen. Finally, after 24 hours the last samples were taken and an NO assay was performed.

1.3 Determination of NO production by RAW 264.7 macrophages after pre-stimulation with LPS, pre-stimulation with IL-10 and co-stimulation of LPS and IL-10.

Once the 24-hour incubation at 37°C of the RAW 264.7 macrophages was completed, the cells were either pre-stimulated with 20 µl IL-10 (30 ng/ml) for two hours, pre-stimulated with 20 µl LPS (100 ng/ml) for 24 hours or co-stimulated with 20 µl with a mixture of both IL-10 (30 ng/ml) and LPS (100 ng/ml) for two hours. For each condition, a sample was collected after two-hour, 24-hour and 26-hour stimulation. At last, an NO assay was performed.

1.4 The effect of pre-stimulation with IL-10 and the peptides on NO production by RAW 264.7 macrophages

After incubation, the RAW 264.7 macrophages were first pre-stimulated with 20 µl of 30 ng/ml IL-10, 30 ng/ml P1, 30 ng/ml P2 and finally with 30 ng/ml P3 for two hours. After this pre-stimulation, 20 µl of 30 ng/ml LPS was added for another 24 hours. After this final incubation, the NO concentrations were determined with an NO assay.

MTT assay

In order to determine the cell viability of the RAW 264.7 cells after treatment with the different cytokines, an MTT assay was performed. This assay was carried out directly after an NO assay was completed, and it was executed according to the MTT assay protocol.
Appendix C: Figure 22

Relative TNF-α expression levels from stimulated RAW 264.7 macrophages

The relative mRNA expression levels of different pro-inflammatory cytokines were determined by performing a qPCR. The RAW 264.7 macrophages ($4*10^5$ cells/well) were first seeded in a 12-wells plate containing 1 ml of medium for 24 hours. Then, 10 µl of IL-10, P1 and P3 were added for one hour followed by an addition of 10 µl of 100 ng/ml LPS for two hours. After this incubation, the stimulated RAW 264.7 cells were harvested and the isolation of mRNA was executed with the Maxwell® 16 LEV simplyRNA Cells Kit. This isolation was done by following the RNA isolation protocol.
Appendix C: Figure 23,24,25,26
Next, the concentration of the isolated mRNA was determined by using the NanoDrop, after which the mRNA was converted into cDNA using the PCR machine and following the PCR machine protocol.
Appendix C
Lastly, the qPCR was prepared by creating a standard curve as well as diluting the cDNA. The cDNA and standard curve were pipetted into a 384 wells plate, after which the Taq Mastermix was added. After this addition, a qPCR was performed according to the PCR protocol. The observed expression levels were compared to the housekeeping gene β-actin. Primers that were used to determine the relative expression were β-actin (forward 5'-ATCGTGCGTGACATCAAAGA-3' and reverse 5'-ATGCCACAGGATTCCATACC-3'), TNF-α (forward 5'-CATCTTCTCAAAATTCGAGTGACAA-3' and reverse 5'-GAGTAGACAAGGTACAACCC-3')
Appendix C: Figure 27

2.1 Determination of relative TNF-α expression by RAW 264.7 macrophages after pre-stimulation with IL-10, pre-stimulation with LPS and co-stimulation.

After 24-hour incubation, the RAW 264.7 macrophages were pre-stimulated with 10 μl of 30 ng/ml IL-10 for one hour followed by addition of 10 μl of 100 ng/ml LPS for two hours. They were also co-stimulated with 10 μl of 30 ng/ml IL-10 and 100 ng/ml LPS for 3 hours.

2.2 The effect of IL-10 and different concentrations of LPS on relative TNF-α expression by RAW 264.7 macrophages.

Once the 24-hour incubation was completed, the macrophages were pre-stimulated with 10 μl of 30 ng/ml IL-10 for one hour followed by addition of 10 μl of 10 ng/ml, 30 ng/ml or 100 ng/ml LPS for two hours. This was done to measure the dose-responsiveness of IL-10 to different doses of LPS. It is important to note that for this experiment, a more recently obtained IL-10 was used.

2.3 Effects of pre-stimulation with IL-10 and the peptides on relative TNF-α expression by RAW 264.7 macrophages.

After finishing the 24-hour incubation, the cells were pre-stimulated with 10 μl of 30 ng/ml IL-10, P1, P2 and P3. Once completed, 10 μl of 30 ng/ml LPS was added for two hours. After the final incubation, a qPCR was performed and compared to the housekeeping gene β-actin.

Results

1.1
Production of nitric oxide by RAW 264.7 macrophages stimulated with LPS, IFN-g and LPS and IFN-g together.

The effect of LPS, IFN-γ and the combination of both on the RAW 264.7 cells was determined by measuring the NO production using an NO assay. The NO concentrations for the different treatments are shown in figure 2. The combination of LPS and IFN-gamma showed the highest increase in NO production in comparison to the control, and increased 1.5-fold compared to the concentration of NO produced from treatment with only 100 ng/ml LPS. Treatment with IFN-γ alone showed no NO production. The control only contained RAW 264.7 macrophages and was not stimulated with LPS or IFN-γ and did not result in production of nitric oxide.

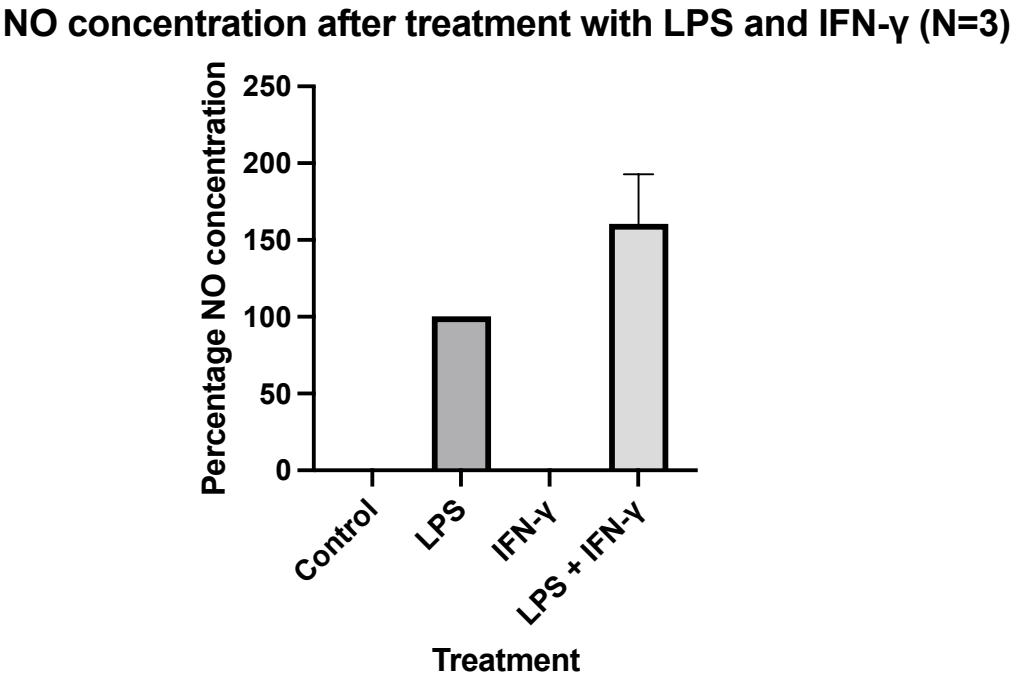

Figure 2 The effect on the NO production of RAW 264.7 macrophages stimulated with either 100 ng/ml LPS, 20 ng/ml IFN-g or 100 ng/ml LPS and 20 ng/ml IFN-g together.

Figure 3 shows the results of the MTT assay performed immediately after the NO assay from figure 2. The cell viability for treatment of RAW 264.7 macrophages with LPS and IFN-γ together showed a slight decrease of 25% compared to the control. The cell viability of the RAW 264.7 macrophages after treatment with solely LPS also showed a slight decrease and treatment with IFN-γ resulted in neither an increase nor a decrease compared to the control. The cell viability for the control is the highest. The control consisted of unstimulated cells and the MTT control did not contain any RAW 264.7 macrophages.

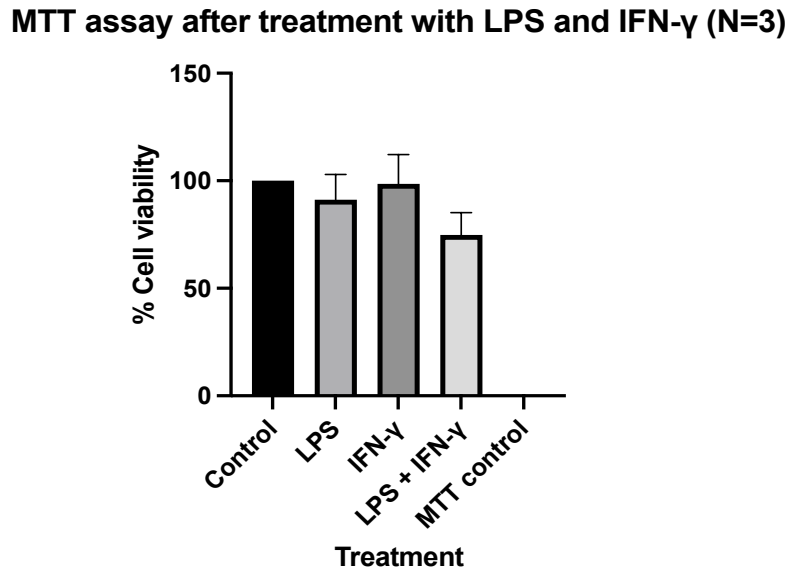

Figure 3 MTT assay corresponding to NO assay from figure 2

1.2 The effect of IL-10, P1, P2 and P3 on the NO concentration in RAW 264.7 macrophages after different time points

To observe the effect of IL-10, P1, P2 and P3 on the NO concentration in RAW 264.7 macrophages, an NO assay was performed. The NO concentrations for the different treatment types and time points can be visualized in figure 4. As can be seen in figure 4, neither IL-10, P1, P2, nor P3 caused a reduction in NO concentration after treatment for all different time points, compared to the control of LPS and IFN-gamma alone. Noteworthy is that in almost all cases, the NO concentration is slightly higher after treatment with IL-10 and the peptides than before they were added. The highest NO concentration was observed after 2-hour incubation of the RAW 264.7 macrophages with P1. Treatment of RAW 264.7 macrophages with P2 for 24 hours did result in a slight decrease in NO concentration. The control consisting of only medium, IL-10 and the peptides alone showed no to very little NO production.

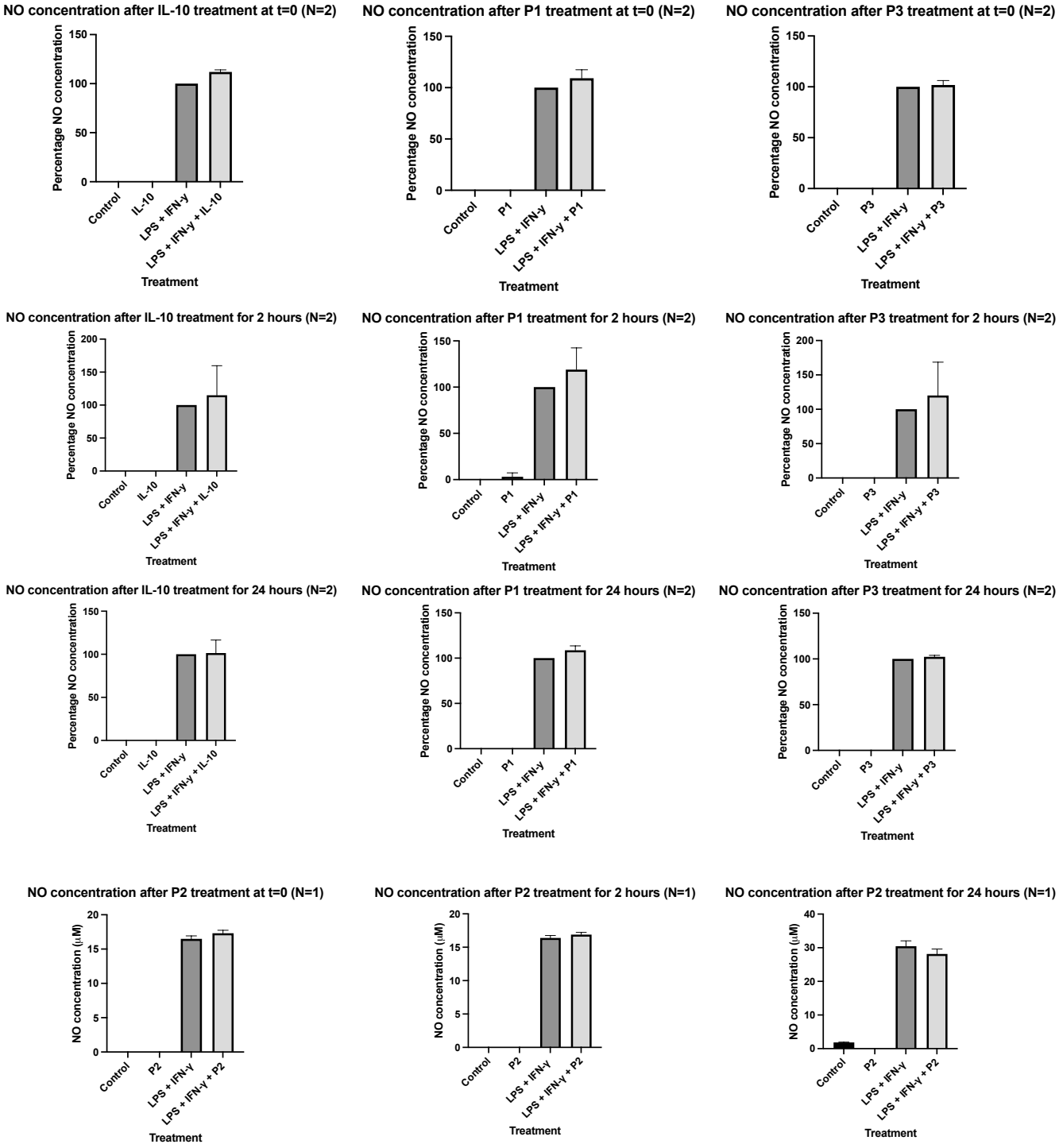

Figure 4 The effect of 30 ng/ml IL-10, 30 ng/ml P1,30 ng/ml P2 and 30 ng/ml P3 stimulation for 0 hours, 2 hours and 24 hours on the NO production in RAW 264.7 cells after 24-hour IFN-gamma and/or LPS treatment. The percentage [NO] (μM) was compared to LPS + IFN-γ to give the final results.

1.3
The effect of IL-10 on nitric oxide production from RAW 264.7 macrophages stimulated with only LPS after different time points and treatment types

To determine the effect of pre-stimulation of RAW 264.7 macrophages with 30 ng/ml IL-10, pre-stimulation with 100 ng/ml LPS and co-stimulation (30 ng/ml IL-10 and 100ng/ml LPS) on the production of NO, an NO assay was performed. The NO assay was performed to visualize the differences in nitric oxide production. The results of the NO assay are shown in figure 5. The highest concentration of NO was observed after 26 hours treatment with pre-stimulation of IL-10. After 24 hours of pre-stimulation with IL-10 and co-stimulation, the NO concentrations slightly decreased for 20% compared to LPS alone. After 26 hours, these NO concentrations increased again, being higher or equal to LPS only after 26 hours. The controls of IL-10 and only medium mostly displayed very little to no NO production.

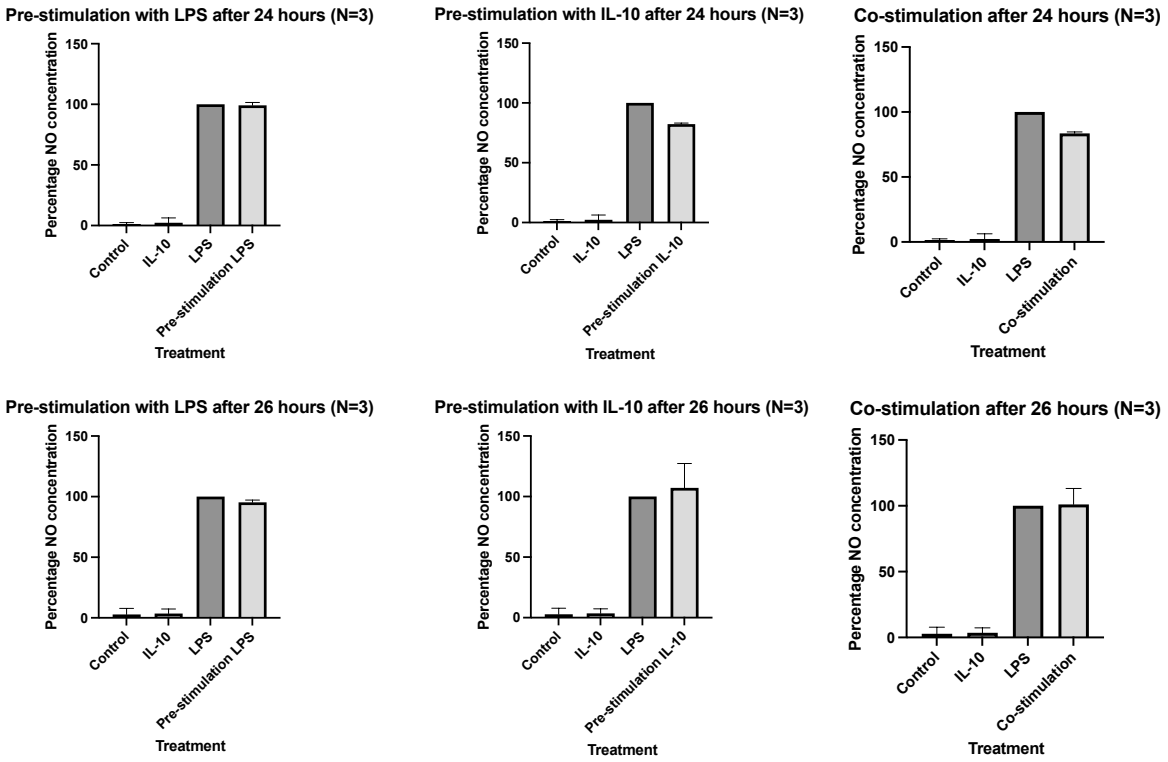

Figure 5 The effect of IL-10 on the produced NO concentrations from RAW 264.7 macrophages after pre stimulation with 30 ng/ml IL-10, pre-stimulation with 100 ng/ml LPS or co-stimulation for, 24 hours and 26 hours. The percentage [NO] (μM) was compared to LPS to give the final results.

2.1
Effects of different treatments with IL-10 on the relative TNF-α expression in RAW 264.7 macrophages

In order to investigate the effect of IL-10 on the relative TNF-α mRNA expression, a qPCR was performed. Figure 6 shows the observed effect on the relative TNF-α expression in RAW 264.7 macrophages after pre-treatment with IL-10 followed by addition of LPS and co-treatment of both IL-10 and LPS. The highest expression of TNF-α was observed in macrophages stimulated with only LPS, whereas pre-treatment of the macrophages with IL-10 showed a reduction of TNF-α expression of approximately 15% compared to treatment of the macrophages with LPS only. Co-stimulation of macrophages with IL-10 and LPS showed no reduction in relative TNF-α expression levels compared to LPS only treatment. The control of IL-10 and only medium also resulted in an increase in relative TNF-α expression levels.

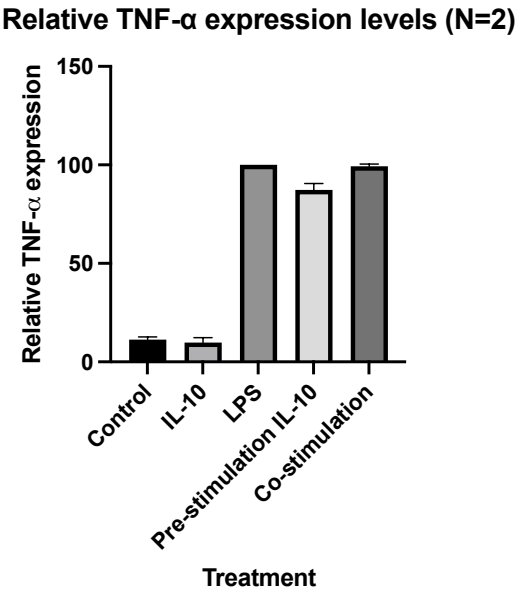

Figure 6 Relative TNF-α expression levels (%) of RAW 264.7 macrophages after pre-stimulation with 30 ng/ml IL-10 or co-stimulation. The results were compared to the housekeeping gene β-actin for determination of the relative expression levels.

2.2
Relative TNF-α expression levels in RAW 264.7 macrophages after treatment with IL-10 and different concentrations of LPS

To further investigate the effect of the more recently obtained IL-10 as well as P1 on the relative TNF-α expression in the macrophages, they were pre-stimulated with IL-10 and P1 for one hour followed by stimulation with LPS. Treatment of the macrophages with any of the LPS concentrations resulted in a large increase in relative TNF-α expression. Treatment of RAW 264.7 macrophages with neither 10 ng/ml, 30 ng/ml nor 100 ng/ml LPS after pre-stimulation with IL-10 does not show a large difference in relative TNF-α expression levels. All relative expression levels decreased after pre-stimulation with IL-10 compared to their respective controls. Pre-treatment with IL-10, followed by 30 ng/ml LPS showed the most reduction in TNF-α expression compared to 30 ng/ml LPS only. Pre-stimulation with 30 ng/ml P1 followed by 100 ng/ml LPS treatment does not show a decrease in relative expression levels, rather it showed an approximate 30% increase in relative TNF-α expression compared to the control of 100 ng/ml LPS. With this result, the last experiment was performed with 30 ng/ml LPS.

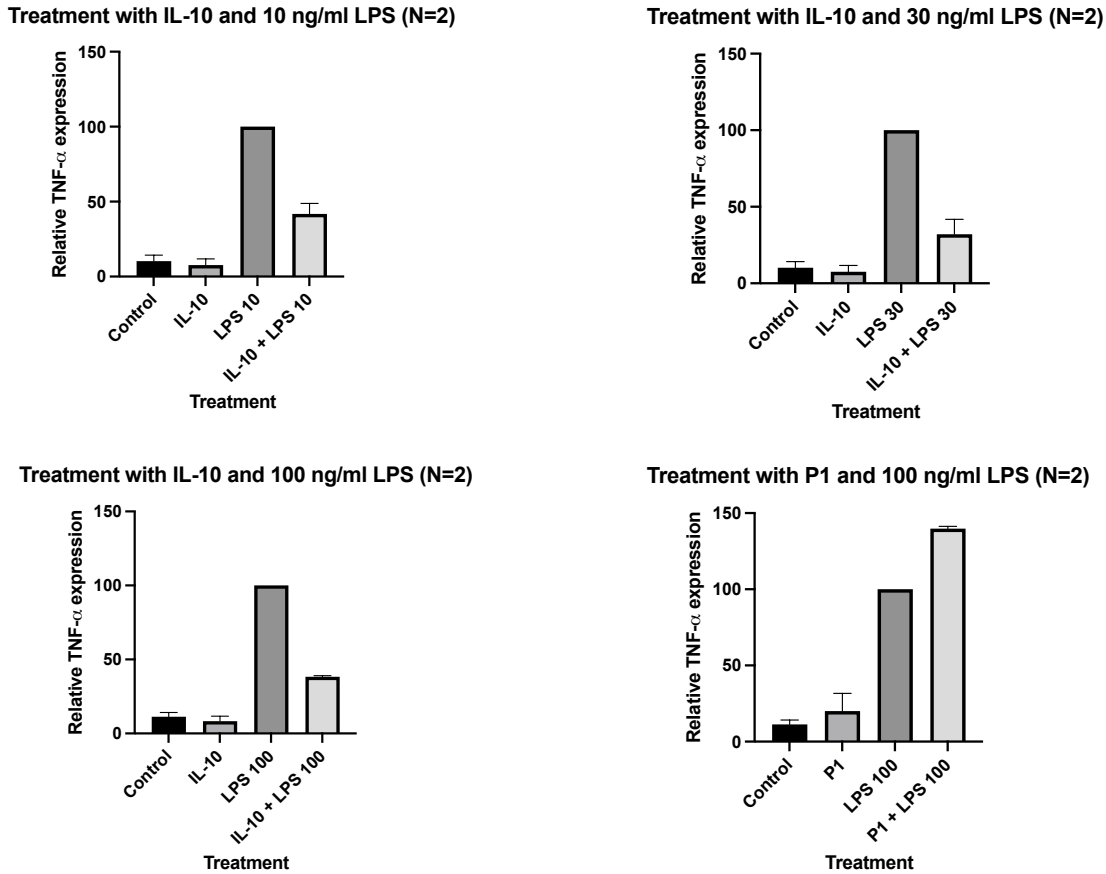

Figure 7 Relative TNF-α expression levels (%) of RAW 264.7 macrophages after treatment with 10 ng/ml, 30 ng/ml and 100 ng/ml LPS. β-actin served as a housekeeping gene to compare the results and to determine the relative expression levels.

2.3
Relative TNF-α expression levels in RAW 264.7 macrophages after pre-stimulation with IL-10 and peptide derivatives

In order to further investigate the anti-inflammatory properties of IL-10, P1, P2 and P3, a qPCR, with the optimal LPS concentration (30 ng/ml) obtained before, was performed. Treatment of macrophages with LPS resulted in a large increase in relative TNF-α expression. Neither pre-stimulation with P1, P2 nor P3 caused a decrease in relative TNF-α expression. In fact, all of them seemed to cause an increase in relative TNF-α expression compared to LPS, with P2 causing the highest increase in expression levels. Pre-stimulation with the more recently obtained IL-10 did however result in a decrease in relative TNF-α expression levels. This decrease is estimated to be approximately two-fold compared to LPS alone.

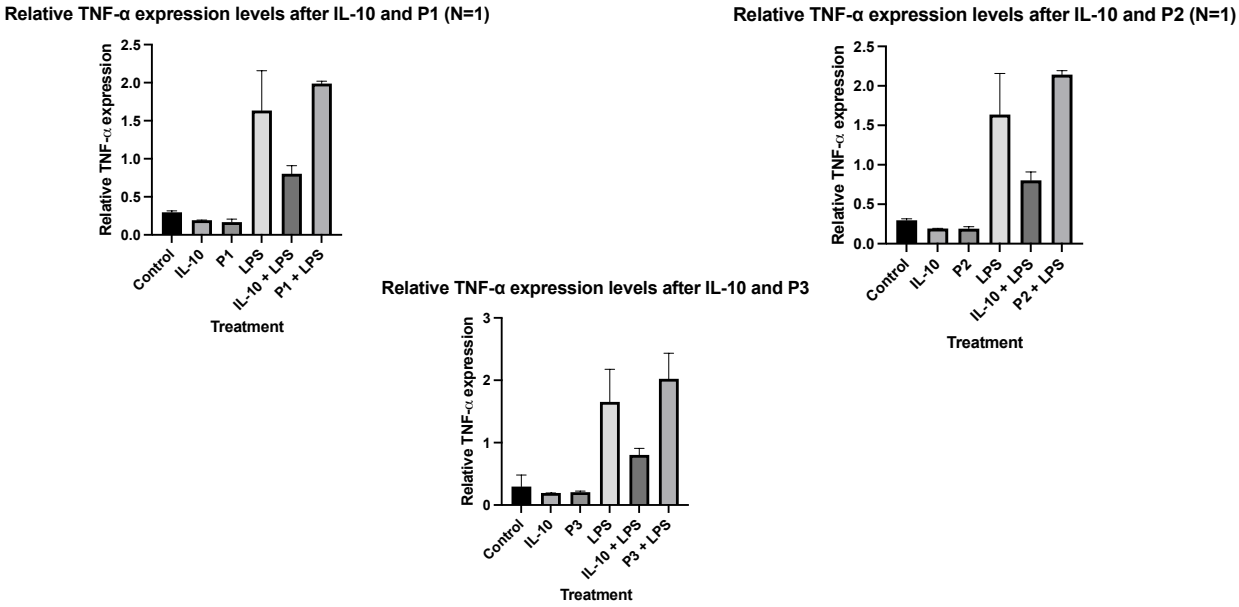

Figure 8 Relative TNF-α expression levels (%) of RAW 264.7 macrophages after pre-stimulation with IL-10, P1, P2 and P3. β-actin served as the housekeeping gene to which the results are compared with.

1.4
Determination of nitric oxide concentration after pre-stimulation with IL-10, P1 and P3

To further investigate the effect of the more recently obtained IL-10, an NO assay was performed. Figure 9 shows the obtained nitric oxide concentrations after pre-stimulation of the RAW 264.7 macrophages with IL-10, P1 and P3, followed by addition of LPS. Treatment with LPS alone caused a large increase in NO production. Only the pre-stimulation of P3 caused a noticeable decrease in NO concentration of approximately 25%, compared to LPS alone. P1 and IL-10 showed no reduction in NO production compared to the control of only LPS. The control contained only RAW 264.7 macrophages without stimulation and displayed no NO concentration. The same is true for the controls of IL-10, P1, P2 and P3.

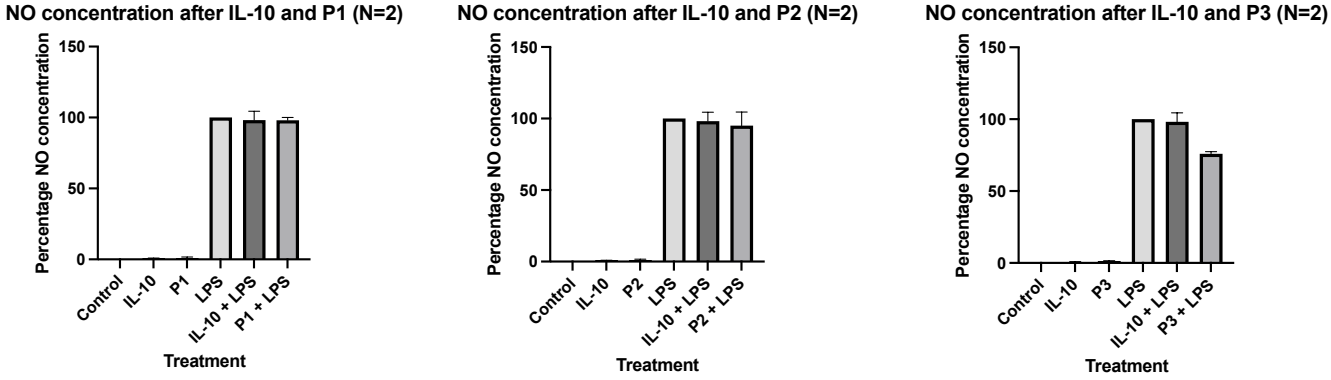

Figure 9 The effect of pre-stimulation with peptide derivatives (30 ng/ml) and newly obtained IL-10 (30 ng/ml) on the NO concentration (%) from RAW 264.7 macrophages stimulated with 30 ng/ml LPS. The percentage [NO] (μM) was compared to LPS (30 ng/ml) to give the final results.

Discussion

The effect of LPS and IFN-gamma on NO production

The results from figure 2 show that stimulation of RAW 264.7 macrophages with LPS causes a large increase in NO production compared to the control of only medium. This is in line with the theory, because once LPS is bound by TLR4 and internalized the macrophages will increase the expression of iNOS. An increased expression of iNOS in the end results in more production of nitric oxide, as can be visualized in figure 2. Treatment of RAW 264.7 macrophages with IFN-gamma and LPS together resulted in an approximate 1.5-fold larger NO production. This is also in line with the expectations, because it is known that IFN-gamma upregulates the TLR4 expression and activity which is bound by LPS for its activation and internalization [11]. The upregulation of TLR4 expression means that more TLR4 is expressed on the surface of the macrophages and this results in an increased uptake of LPS. This increased uptake of LPS then causes an even higher expression of iNOS and other inflammatory cytokines, which in the end results in more NO being produced than without the presence of IFN-γ. IFN-γ alone showed no NO production, which is also known to be true because IFN-gamma on its own only has an indirect effect on the NO production and no direct effect on the NO production. This indirect effect is the upregulation of TLR4 expression, as mentioned above [11].
The MTT assay as seen in figure 3 shows a 25% decrease in cell viability after treatment with LPS or IFN-gamma. The cell viability for the control is the highest, as expected. It is expected that a decrease in cell viability should lead to a decrease in NO production. In this case, one can see in figure 2 that the NO concentration is highest for LPS and IFN-γ treatment. The reason for this is as described above; more LPS is internalized because of the upregulated TLR4 expression. This means that more NO is produced, despite the cell viability decreasing. This means that one can conclude that the concentrations used for LPS and IFN-γ do not significantly affect cell viability of RAW 264.7 macrophages. Thus, changes in NO production cannot be explained by changes in cell viability.

The effect of IL-10, P1, P2 and P3 on NO production by RAW 264.7 macrophages

After the results from the first NO assay were obtained and indicated that the combination of LPS and IFN-γ resulted in the highest production of NO, a new NO assay was performed. In this case, IL-10 and the peptides were added to determine their inflammatory effects. As can be visualized from figure 3, treatment of RAW 264.7 macrophages with LPS and IFN-γ caused a large increase in NO production compared to the control. This again, is expected. The anti-inflammatory effect of IL-10 on macrophages functions on the induction of SOCS3. SOCS3 is known to inhibit iNOS expression and thus cause a reduction in NO production [8]. Thus, it would be expected that treatment of RAW 264.7 macrophages with IL-10 results a noticeable decrease in nitric oxide production. However, in none of the cases for IL-10 and the peptides, a decrease in NO concentration is observed. In fact, in almost all cases an increase in NO concentration is observed. Except for P2, which was the only cytokine to have displayed a slight reduction in NO concentration after 24 hours of stimulation. This still however, contradicts the expectation of observing the anti-inflammatory effect of IL-10, but it is in line with the expectation to observe pro-inflammatory effects of the peptides.

A theory for this observation could be that the combination of LPS and IFN-γ results in an inflammatory response that cannot be turned around by IL-10 nor the peptides. This would be the case because as mentioned before, with IFN-γ upregulating the TLR4 expression. This constant upregulation of TLR4 together with LPS being added prior to IL-10 suggests that there is too much inflammation occurring in comparison to the anti-inflammatory effects of IL-10 and its derivatives. This is due to IFN-γ inhibiting endogenous IL-10 production while also simultaneously increasing TNF-α expression, thus increasing the inflammation [12]. This could mean that the majority of macrophages would still be M1 macrophages and that the minority of the macrophages would be the anti-inflammatory M2 macrophages. This theory then explains the reason for not being able to observe the anti-inflammatory effect of IL-10 and the peptides.
It is also important to note that these results are not significant, because the experiment has only been performed twice for P1 and P3 (N=2) and once for P2 (N=1). For this reason and the contradiction of the hypothesis, it is recommended to perform this experiment at least once more to make the results significant.

Difference in NO concentration after pre-stimulation with IL-10, LPS and co-stimulation

Due to the reasoning described above, another NO assay was performed. This time, IL-10 or LPS were added prior to one another, and co-stimulation of the two was tested. As can be observed in figure 4, after 24 hours and 26 hours of stimulation with LPS prior to IL-10, the NO concentration significantly increased by a large amount. After 24 hours of pre-stimulation with IL-10 and co-stimulation, a slight decrease in NO concentration of approximately 20% compared to LPS alone can be observed. This is expected, because as mentioned before, addition of IL-10 to RAW 264.7 macrophages polarizes them into M2 macrophages. This polarization into M2 means that the macrophages display anti-inflammatory properties. These properties, together with IL-10's effect on SOCS3, explains the observed reduction in nitric oxide concentration. Pre-stimulation with LPS prior to IL-10 addition showed no reduction in NO concentration again. This can be explained by the same reasoning as mentioned above, which states that when adding LPS prior to IL-10, the majority of macrophages will still be M1. After another two hours, the nitric oxide concentrations increased for all treatment types and are higher than the NO concentration produced by LPS alone. This is contradicting the results of 24 hours, indicating that IL-10 has lost its anti-inflammatory activity. Since the loss of the anti-inflammatory effects of IL-10 after 26 hours of stimulation is significant (N=3), it is suggested that if one wants to determine these effects of IL-10, one should find the NO concentration for a lower stimulation time of stimulation with IL-10 and LPS. It could be that the optimal time of stimulation with LPS, in order for IL-10 to exert its anti-inflammatory effect properly, lies lower than 24 hours since the decrease in NO production was observed at 22 hours of stimulation. Based on the varying results regarding the NO assays, it was concluded that determination of nitric oxide was not a suitable parameter to determine the inflammatory effects of IL-10 and the peptides.

Effect of IL-10 on relative TNF-α expression levels

Due to the conclusion that determination of NO was not a suitable parameter to determine the inflammatory effects of the peptides and IL-10, a new method was performed. The effect of IL-10 on relative TNF-α expression was tested by performing a qPCR. The stimulation of RAW 264.7 macrophages with 100 ng/ml LPS for two hours resulted in a large increase in relative TNF-α expression levels compared to the control containing only medium. This is expected, because as stated before once LPS binds to TLR4, it gets activated. This activation will result in the activation of the MyD88-dependent and TRIF-dependent signaling pathways. These signaling pathways are responsible for the activation of NF-kβ and MAPK, which in the end causes the production of pro-inflammatory cytokines like TNF-α [7]. Ultimately, as a result of the release of pro-inflammatory cytokines, the MHC-II expression in LPS-stimulated RAW 264.7 macrophages also increases. In figure 5, one can observe this effect as TNF-α expression levels were about ten times larger than for the control.
As mentioned above, pre-stimulation with IL-10 should in theory should lead to lower expression levels of TNF-α due to its anti-inflammatory properties. As can be seen in figure 5, there was a slight reduction of approximately 15% in TNF-α expression levels after pre-stimulation with IL-10. This is also expected, as IL-10 prevents LPS-induced augmentation of the MyD88-signaling pathway, which is responsible for TNF-α production. It also causes the polarization of M0 or M1 macrophages into M2 macrophages. This polarization into M2 would suggest that less TNF-α is produced, as was the case for figure 5 [9,10]. Co-stimulation of IL-10 and LPS however does not show any decrease in relative TNF-α expression levels, which is in contrast to the expectations. The obtained expression levels are the same for the control of LPS and co-stimulation. This result is caused by LPS being able to trigger the MyD88 expression before IL-10 would be able to down-regulate the expression of MyD88 [13]. This would then explain not being able to observe a decrease in relative TNF-α expression levels after co-stimulation. Thus, since the decrease in TNF-α expression levels after pre-stimulation with IL-10 were not as large as expected and co-stimulation does not result in a decrease in expression levels and the experiment has only been performed twice, it is suggested to execute the same experiment at least once more in order to define the obtained results as significant.

Effect of IL-10 and different LPS concentrations on relative TNF-α expression levels

Since the reduction in TNF-α expression levels after pre-stimulation with IL-10 followed by 100 ng/ml LPS was not as high as expected, different dosages of LPS were tested to find an optimal concentration of LPS for which IL-10 can exert its anti-inflammatory effect. As can be seen from figure 7, 10 ng/ml LPS, 30 ng/ml LPS and 100 ng/ml LPS caused a great increase in TNF-α expression levels, which was expected [7]. Pre-treatment of IL-10, followed by addition of 30 ng/ml LPS resulted in the greatest reduction in TNF-α expression levels. It is important to note that for this experiment, a more recently obtained IL-10 had been used. It can be seen from figure 7 that even though the LPS concentrations differ, all relative TNF-α expression levels seem to decrease by a large amount. Specifically comparing the reduction in TNF-α expression levels after 100 ng/ml LPS of the previous experiment and this experiment, one can observe that IL-10 had a much greater effect in the reduction in relative TNF-α expression levels.

This might suggest that more recently synthesized IL-10 has greater anti-inflammatory effects than older IL-10. For this reason, the next experiments with this new IL-10 have been performed. Pre-stimulation with P1 resulted in an increase in relative TNF-α expression levels, suggesting that it has pro-inflammatory properties.
This is expected as P1 should in theory be pro-inflammatory. The results of this experiment are not significant however (N=2), which means that it is advised to perform this experiment with the same conditions at least once more.

Relative TNF-α expression levels after pre-stimulation with IL-10, P1, P2 and P3

Since the optimal concentration of LPS to stimulate the macrophages turned out to be 30 ng/ml, another qPCR had been performed. The qPCR data from figure 7 shows that after stimulation of RAW 264.7 macrophages with 30 ng/ml LPS for 2 hours, the relative TNF-α expression levels increased by a large amount compared to the control. This is again in line with the theory. It is noteworthy that pre-stimulation with all peptides does not show any reduction in relative TNF-α expression levels. Rather, the results display that the TNF-α expression levels were higher for pre-stimulation with the peptides than LPS alone. This is in line with the theory, suggesting that the peptides do not show anti-inflammatory properties but rather pro-inflammatory properties. On the basis of earlier obtained results, this might be true. In all performed experiments containing the peptides, they mostly did not result in a reduction in NO production nor relative TNF-α expression levels when treated with LPS. Instead, they caused an increase in both nitric oxide and relative TNF-α expression levels thus suggesting that they contain pro-inflammatory properties. However, the results of this experiment are not significant (N=1), which indicates that it is suggested to at least perform this experiment twice more.

NO concentration after pre-stimulation with IL-10 and P1, P2 and P3

The effect of the newly obtained IL-10 was also determined with an NO assay. The results from figure 9 show that treatment of macrophages with LPS resulted in a large increase in NO concentration again. The nitric oxide concentration was not lowered after pre-stimulation with IL-10. Peptide 1 and 2 also did not cause a large increase nor decrease in NO concentration after pre-stimulation. P3 however, did show a reduction after pre-stimulation of around 25% relative to the control. Since the peptides are derivatives and short parts of IL-10, P3 could have obtained the part of IL-10 that exerts its anti-inflammatory properties. This result does however contradict the earlier obtained results, because P3 did not display any anti-inflammatory effects in an NO assay before. An explanation for this observation can be that in this experiment, 30 ng/ml LPS was used instead of 100 ng/ml LPS. This could mean that P3 is more able to exert its anti-inflammatory effect on the RAW 264.7 macrophages since there is less LPS present due to the lower concentration. If less LPS is present due to the lower concentration, it would mean that ultimately less NO is produced. Since the concentrations of IL-10 and the peptides remained the same throughout all experiments, it is possible that P3 is now able to display its anti-inflammatory properties in this NO assay. Another important thing to note is that P3 did result in an increase in TNF-α expression, suggesting its pro-inflammatory properties.

Having obtained the result that shows P3 causing a decrease in NO concentration contradicts the result on TNF-α expression, because this suggests that P3 might be able to reduce NO via anti-inflammatory properties.
Since the results from this experiment are also not significant (N=2), it is suggested to perform the same experiment at least once more in order to confirm the inflammatory effect of P3.

A noticeable observation regarding the results from all NO assays is that over time, higher NO concentrations were measured. The last NO assay performed with the same cell line with passage number 8 resulted in twice as large (40 μM) nitric oxide being produced compared to the first NO assay performed (20 μM).
This large increase in NO production is due to the acidic environment of the RAW 264.7 macrophages. RAW 264.7 macrophages remain stable through passage number 10 up to 30 [14]. The passage number used for the final experiment was 36 (p8 + 26). It was decided to continue with this passage number, because the study only lasted one more week. However, this passage number is well beyond 30. It was observed that the medium containing the macrophages turned acidic much faster during incubation at 37°C, suggesting that their metabolism had been changed. The change into more acidic medium also has an effect on the iNOS activity from RAW 264.7 macrophages in response to LPS. When the pH of the environment of RAW 264.7 macrophages lowers, the activity of iNOS will be upregulated [15]. This means that even though a lower concentration of LPS was used in the last experiment, more NO will be produced because of this upregulated activity of iNOS. These results were seen over time during this study.

Further research

An important thing to note is that almost every result obtained from the NO assays about the anti-inflammatory effects of IL-10 is not in line with the theory. The results of the peptides are sometimes in line with the theory, but are also not in line a few times. This means that the results are rather inconsistent, since some results show that the peptides do cause a reduction in nitric oxide production and some show that they do not. This suggests that determining NO concentrations might not be a suitable parameter in order to determine these effects of IL-10 and its derivatives. Instead, it is suggested to perform more qPCR experiments and determine the effects of the peptides and IL-10 in this manner. Since the results of the anti-inflammatory effects of IL-10 show that it decreases TNF-α expression levels and especially the peptide derivatives causing large increases in TNF-α expression, it is also suggested that the peptides do not display these anti-inflammatory effects at all. This would mean that the peptide derivatives have pro-inflammatory effects and could possibly also have anti-fibrotic effects, since these peptides are part of the IL-10 molecule and IL-10 displays both anti-inflammatory and ant-fibrotic effects [16]. Further research on these peptides should thus focus on the anti-fibrotic effects of IL-10 and the peptide derivatives. This can be done by determining the IL-10R expression on either RAW 264.7 macrophages or fibroblasts with immunohistochemistry, immunofluorescence microscopy or PCR analysis of the receptors. Another method to determine the anti-fibrotic effects of IL-10 and the peptides is to determine the expression of transforming growth factor-β1 (TGF-β1), matrix metalloproteinase 1 (MMP1) and MMP8. IL-10 is known to down-regulate TGF-β1 and upregulate MMP1 and MMP8 [17].
Determination of these expressions after treatment with IL-10 and the peptide derivatives could possibly uncover the anti-fibrotic effects of both IL-10 and the peptides.

Conclusion

In conclusion, from the obtained results one can conclude that when stimulating RAW 264.7 macrophages with LPS, a large difference in NO concentration can be observed. Furthermore, there is also a large increase in relative TNF-α mRNA expression. When stimulating the macrophages with LPS and IFN-γ together, an approximate 1.5-fold larger NO response is measured. There is no real difference in relative TNF-α mRNA expression after treatment with LPS alone or with LPS and IFN-γ together. However, it was determined that IL-10 did not have a large effect on the relative TNF-α mRNA expression after treatment with both LPS and IFN-γ. The anti-inflammatory effect of IL-10 was only observed after treatment of RAW 264.7 macrophages with only LPS. IL-10 greatly lowered relative TNF-α mRNA expression. It was also seen that IL-10 did not show large effects on the reduction in nitric oxide production, except for pre-treatment with IL-10 and co-treatment with IL-10 and LPS after 22 hours. In these cases, IL-10 did slightly reduce the nitric oxide concentration, but these concentrations increased again after two hours. Regarding the inconsistent results of the observed effects of IL-10 from the NO assays, it can be concluded that measuring nitric oxide production is not a suitable parameter to determine the inflammatory effects of IL-10. Instead, it was concluded that determining relative TNF-α mRNA expression is a good parameter to determine the anti-inflammatory effect of IL-10, because these results are consistent throughout this entire study.
The peptide derivatives of IL-10 did not have reducing effects on the produced nitric oxide. Neither pre-stimulation with the peptides, nor with LPS resulted in a reduction in NO concentration. However, P3 did show a slight reduction in NO concentration after pre-stimulation with P3, but this result is not significant. The peptide derivatives also did not display any reducing effects of relative TNF-α mRNA expression. Most of the time, the relative TNF-α mRNA expression increased after treatment with the peptides. This suggests that they do not display the anti-inflammatory effects of IL-10, but rather display pro-inflammatory effects. From the obtained results with the peptides, it can be concluded that they could potentially be effective as a pro-inflammatory substance in the treatment of liver fibrosis and might also be used as an anti-fibrotic after further research has been performed. Since all results from the qPCR were consistent and greatly displayed the inflammatory effects of IL-10 and the peptides, it can be concluded that the qPCR is the optimal method to determine the inflammatory effects of IL-10 and the peptide derivatives.

As no significant inflammatory effect of IL-10 and the peptides were determined with the NO assays, and the NO concentration is not a good parameter to determine the inflammatory activity of IL-10 and the peptides, further studies on this topic should focus more on qPCR experiments to determine these inflammatory effects. One should also focus on uncovering the anti-fibrotic effects of the peptides. This can be done by first determining the IL-10 receptor expression as well as determining TGF-β1, MMP1 and MMP8 expression levels after treatment.

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Appendix

Appendix A - Determination of NO concentration in stimulated RAW 264.7 macrophages

1.1 Raw data corresponding to figure 2 and 3

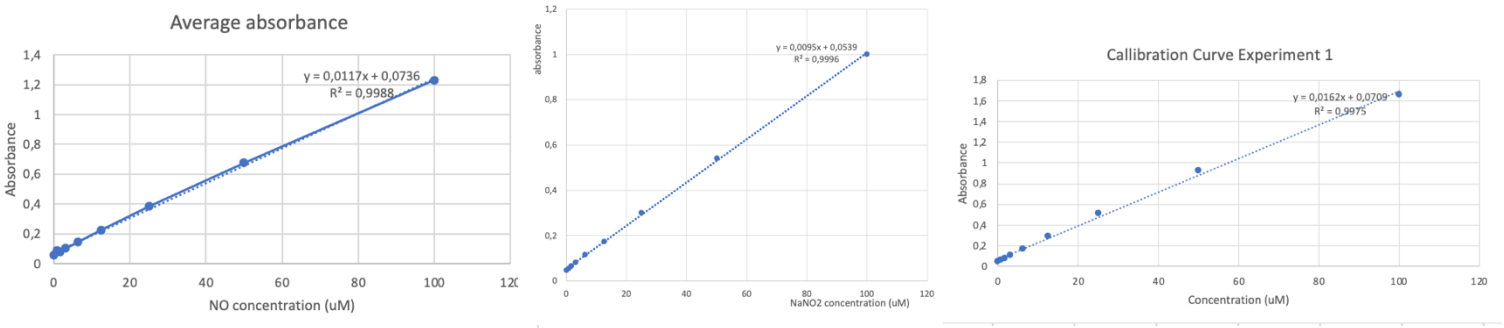


Figure 10 The calibration curves corresponding to the NO assay from figure 2. From left to right; R^2 = 0,9988, R^2 = 0,999, R^2 = 0,9975

Table 1 The raw data from the NO assay corresponding to figure 2

Treatment	*Absorbance*		
Control	0,053	0,053	0,051
LPS	0,199	0,052	0,204
IFN-γ	0,051	0,054	0,052
LPS + IFN-γ	0,306	0,295	0,300
Control	0,056	0,063	0,054
LPS	0,185	0,253	0,174
IFN-γ	0,059	0,059	0,058
LPS + IFN-γ	0,279	0,283	0,296
Control	0,060	0,052	0,058
LPS	0,174	0,229	0,187
IFN-γ	0,053	0,053	0,060
LPS + IFN-γ	0,219	0,232	0,227
Treatment	*Average absorbance*	*Concentration (μM)*	*Concentration + std (μM)*
Control	0,0523	-1,82	-1,82 ± -0,04
LPS	0,2015	10,93	10,93 ± 4,68
IFN-γ	0,052	-1,85	-1,85 ± -0,062
LPS + IFN-γ	0,3003	19,38	19,38 ± 0,355
Control	0,055	0,1	0,1 ± 0,001
LPS	0,180	13,2	13,2 ± 0,008
IFN-γ	0,059	0,5	0,5 ± 0,001
LPS + IFN-γ	0,281	23,8	23,8 ± 0,003
Control	0,057	-0,878	-0,878 ± -0,064
LPS	0,197	7,763	7,763 ± 1,14
IFN-γ	0,055	-0,961	-0,961 ± -0,070
LPS + IFN-γ	0,226	9,574	9,574 ± 0,277

Table 2 Data corresponding to the MTT assay from figure 3

Treatment	*Absorbance*		
Control	0,677	0,525	0,493
LPS	0,684	0,825	0,596
IFN-γ	0,992	0,595	0,809
LPS + IFN-γ	0,688	0,452	0,504
MTT control	0,099	0,1	0,103
Control	0,605	0,590	0,414
LPS	0,271	0,603	0,429
IFN-γ	0,378	0,516	0,528
LPS + IFN-γ	0,479	0,384	0,564
MTT control	0,086	0,072	0,067
Control	0,410	0,674	0,569
LPS	0,549	0,547	0,550
IFN-γ	0,584	0,474	0,534
LPS + IFN-γ	0,407	0,415	0,406
MTT control	0,086	0,096	0,088

Table 3 Data corresponding to the MTT assay from figure 3

Treatment	*Viability*			*Average percentage*	*Average percentage ± STD*
Control	0,919	0,650	0,594	72,11	72,11 ± 7,080
LPS	0,932	1,181	0,776	96,31	96,31 ± 28,06
IFN-γ	1,477	0,774	1,153	113,47	113,47 ± 49,97
LPS + IFN-γ	0,934	0,521	0,613	69,11	69,11 ± 27, 68
MTT control	-0,1036	-0,1029	-0,0965	0	0 ± 0
Control	0,605	0,590	0,414	53,63	100 ± 19,80
LPS	0,271	0,603	0,429	77,89	77,89 ± 29,78
IFN-γ	0,378	0,516	0,528	86,49	86,49 ± 15,21
LPS + IFN-γ	0,479	0,384	0,564	86,85	86,85 ± 16,44
MTT control	0,086	0,072	0,075	0,075	0,075 ± 0
Control	0,32	0,584	0,479	100	100 ± 28,83
LPS	0,459	0,457	0,460	99,50	99,50 ± 0,33
IFN-γ	0,494	0,384	0,444	95,60	95,60 ± 11,95

LPS + IFN-γ	0,317	0,325	0,316	68,70	68,70 ± 0,150
MTT control	0,00	0,00	0,00	0	0 ± 0

1.2 Raw data from figure 4

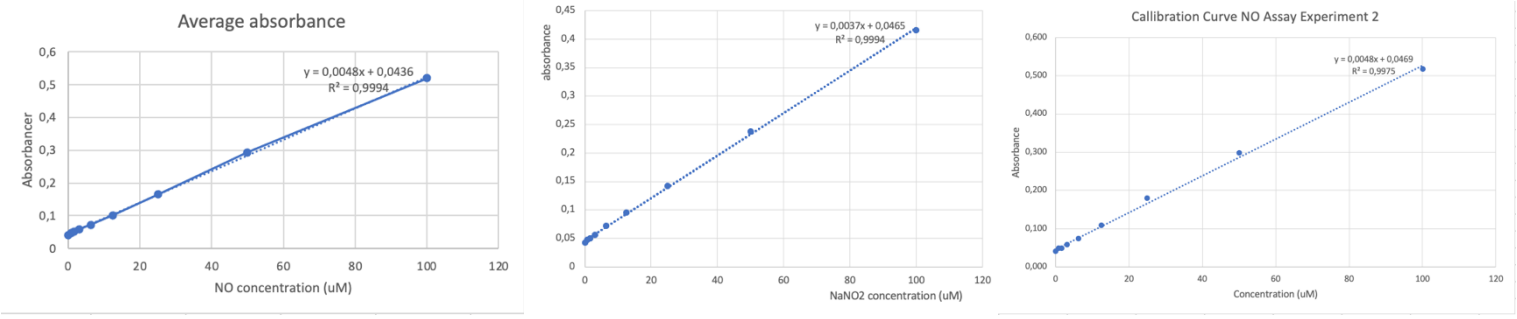


Figure 11 Calibration curves corresponding to the NO assay in figure 4. From left to right: R^2 = 0,9994, R^2 = 0,9994, R^2 = 0,9975.

Table 4 Data obtained from NO assay from figure 4

Treatment	*Time (h)*	*Absorbance*		
Control	0	0,047	0,047	0,062
LPS + IFN-γ	0	0,106	0,15	0,141
IL-10	0	0,043	0,048	0,043
P1	0	0,047	0,052	0,046
P3	0	0,045	0,043	0,045
LPS + IFN-γ + IL-10	0	0,127	0,148	0,150
LPS + IFN-γ + P1	0	0,138	0,154	0,145
LPS + IFN-γ + P3	0	0,124	0,135	0,151
Control	0	0,046	0,045	0,049
LPS + IFN-γ	0	0,133	0,155	0,150
IL-10	0	0,042	0,042	0,042
P1	0	0,043	0,043	0,044
P3	0	0,045	0,044	0,046
LPS + IFN-γ + IL-10	0	0,135	0,171	0,172
LPS + IFN-γ + P1	0	0,141	0,144	0,163
LPS + IFN-γ + P3	0	0,129	0,152	0,153
Control	0	0,045	0,045	0,048
LPS + IFN-γ	0	0,092	0,106	0,110
IL-10	0	0,047	0,044	0,046
P1	0	0,046	0,045	0,044
P2	0	0,046	0,045	0,045
LPS + IFN-γ + IL-10	0	0,097	0,103	0,113
LPS + IFN-γ + P1	0	0,102	0,106	0,111
LPS + IFN-γ + P2	0	0,113	0,126	0,109
Control	2	0,047	0,040	0,301

LPS + IFN-γ	2	0,092	0,112	0,107
IL-10	2	0,039	0,040	0,047
P1	2	0,042	0,040	0,050
P3	2	0,050	0,039	0,043
LPS + IFN-γ + IL-10	2	0,139	0,124	0,132
LPS + IFN-γ + P1	2	0,097	0,149	0,129
LPS + IFN-γ + P3	2	0,130	0,136	0,143
Control	2	0,044	0,041	0,047
LPS + IFN-γ	2	0,141	0,192	0,162
IL-10	2	0,042	0,043	0,046
P1	2	0,040	0,044	0,078
P3	2	0,045	0,047	0,046
LPS + IFN-γ + IL-10	2	0,131	0,156	0,149
LPS + IFN-γ + P1	2	0,141	0,157	0,205
LPS + IFN-γ + P3	2	0,138	0,155	0,152
Control	2	0,047	0,046	0,046
LPS + IFN-γ	2	0,109	0,105	0,109
IL-10	2	0,053	0,046	0,046
P1	2	0,049	0,045	0,045
P2	2	0,045	0,046	0,043
LPS + IFN-γ + IL-10	2	0,149	0,113	0,130
LPS + IFN-γ + P1	2	0,104	0,100	0,098
LPS + IFN-γ + P2	2	0,111	0,122	0,108
Control	24	0,045	0,043	0,057
LPS + IFN-γ	24	0,165	0,186	0,184
IL-10	24	0,049	0,052	0,052
P1	24	0,049	0,058	0,054
P3	24	0,056	0,060	0,057
LPS + IFN-γ + IL-10	24	0,196	0,191	0,197
LPS + IFN-γ + P1	24	0,188	0,195	0,201
LPS + IFN-γ + P3	24	0,181	0,179	0,189
Control	24	0,056	0,042	0,056
LPS + IFN-γ	24	0,174	0,193	0,203
IL-10	24	0,042	0,043	0,044
P1	24	0,043	0,041	0,043
P3	24	0,046	0,042	0,043
LPS + IFN-γ + IL-10	24	0,150	0,183	0,197
LPS + IFN-γ + P1	24	0,172	0,207	0,214
LPS + IFN-γ + P3	24	0,174	0,196	0,205
Control	24	0,050	0,054	0,056

LPS + IFN-γ	24	0,195	0,166	0,154
IL-10	24	0,047	0,046	0,047
P1	24	0,044	0,044	0,044
P2	24	0,046	0,045	0,047
LPS + IFN-γ + IL-10	24	0,182	0,169	0,257
LPS + IFN-γ + P1	24	0,177	0,157	0,150
LPS + IFN-γ + P2	24	0,174	0,157	0,146

Table 5 Data obtained from NO assay from figure 3

Treatment	*Time (h)*	*Average absorbance*	*Concentration (μM)*	*Concentration ± std (μM)*
Control	0	0,52	1,75	1,75 ± 0,2914
LPS + IFN-γ	0	0,132	18,49	18,49 ± 3,247
IL-10	0	0,045	0,22	0,22 ± 0,014
P1	0	0,048	0,986	0,986 ± 0,065
P3	0	0,044	0,153	0,153 ± 0,004
LPS + IFN-γ + IL-10	0	0,142	20,43	20,43 ± 1,837
LPS + IFN-γ + P1	0	0,146	21,26	21,26 ± 1,171
LPS + IFN-γ + P3	0	0,137	19,39	19,39 ± 1,926
Control	0	0,0467	-0,0486	-0,0486 ± 0
LPS + IFN-γ	0	0,146	20,64	20,64 ± 1,63
IL-10	0	0,042	-1,02	-1,02 ± 0
P1	0	0,0433	-0,743	-0,743 ± 0
P3	0	0,045	-0,395	-0,395 ± 0
LPS + IFN-γ + IL-10	0	0,1593	23,42	23,42 ± 3,09
LPS + IFN-γ + P1	0	0,1493	21,34	21,34 ± 1,704
LPS + IFN-γ + P3	0	0,1446	20,36	20,36 ± 1,911
Control	0	0,046	-0,10	-0,10 ± -0,005
LPS + IFN-γ	0	0,108	16,50	16,50 ± 1,443
IL-10	0	0,046	-0,20	-0,20 ± -0,008
P1	0	0,045	-0,40	-0,40 ± -0,009
P2	0	0,045	-0,40	-0,40 ± -0,004
LPS + IFN-γ + IL-10	0	0,104	15,5	15,5 ± 1,202
LPS + IFN-γ + P1	0	0,106	16,0	16,0 ± 0,681
LPS + IFN-γ + P2	0	0,111	17,30	17,30 ± 0,441
Control	2	0,129	17,86	17,86 ± 20,54
LPS + IFN-γ	2	0,104	12,51	12,51 ± 1,256
IL-10	2	0,042	-0,33	-0,33 ± 0,035
P1	2	0,044	0,083	0,083 ± 0,100
P3	2	0,044	0,083	0,083 ± 0,105

LPS + IFN-γ + IL-10	2	0,132	18,35	18,35 ± 1,046
LPS + IFN-γ + P1	2	0,125	16,96	16,96 ± 3,559
LPS + IFN-γ + P3	2	0,136	19,32	19,32 ± 0,922
Control	2	0,044	-0,604	-0,604 ± 0
LPS + IFN-γ	2	0,165	24,604	24,604 ± 3,822
IL-10	2	0,0436	-0,673	-0,673 ± 0
P1	2	0,054	1,479	1,479 ± 0,572
P3	2	0,046	-0,188	-0,188 ± 0
LPS + IFN-γ + IL-10	2	0,145	20,51	20,51 ± 1,82
LPS + IFN-γ + P1	2	0,167	25,16	25,16 ± 4,99
LPS + IFN-γ + P3	2	0,148	21,13	21,13 ± 1,29
Control	2	0,046	-0,045	-0,045 ± -0,0005
LPS + IFN-γ	2	0,108	16,50	16,50 ± 0,355
IL-10	2	0,048	0,50	0,50 ± 0,0414
P1	2	0,046	-0,045	-0,045 ± -0,002
P2	2	0,045	-0,50	-0,50 ± -0,017
LPS + IFN-γ + IL-10	2	0,131	22,70	22,70 ± 3,135
LPS + IFN-γ + P1	2	0,099	14,20	14,20 ± 0,438
LPS + IFN-γ + P2	2	0,110	17,00	17,00 ± 1,146
Control	24	0,048	0,986	0,986 ± 0,154
LPS + IFN-γ	24	0,178	28,07	28,07 ± 1,824
IL-10	24	0,051	1,542	1,542 ± 0,052
P1	24	0,054	2,097	2,097 ± 0,176
P3	24	0,058	2,931	2,931 ± 0,106
LPS + IFN-γ + IL-10	24	0,195	31,47	31,47 ± 0,519
LPS + IFN-γ + P1	24	0,195	31,47	31,47 ± 1,052
LPS + IFN-γ + P3	24	0,183	29,04	29,04 ± 0,840
Control	24	0,0513	0,924	0,924 ± 0,145
LPS + IFN-γ	24	0,190	29,81	29,81 ± 2,311
IL-10	24	0,043	-0,812	-0,812 ± 0
P1	24	0,0423	-0,951	-0,951 ± 0
P3	24	0,0436	-0,674	-0,674 ± 0
LPS + IFN-γ + IL-10	24	0,177	27,03	27,03 ± 3,69
LPS + IFN-γ + P1	24	0,198	31,41	31,41 ± 3,58
LPS + IFN-γ + P3	24	0,192	30,16	30,16 ± 0,70
Control	24	0,053	1,80	1,80 ± 0,106
LPS + IFN-γ	24	0,160	30,70	30,70 ± 4,041
IL-10	24	0,047	0,045	0,045 ± 0,001

P1	24	0,044	-0,70	-0,70 ± 0
P2	24	0,046	-0,10	-0,10 ± -0,003
LPS + IFN-γ + IL-10	24	0,176	34,90	34,90 ± 9,437
LPS + IFN-γ + P1	24	0,154	28,90	28,90 ± 2,640
LPS + IFN-γ + P2	24	0,152	28,40	28,40 ± 2,642

1.3 Raw data used to obtain results in figure 5

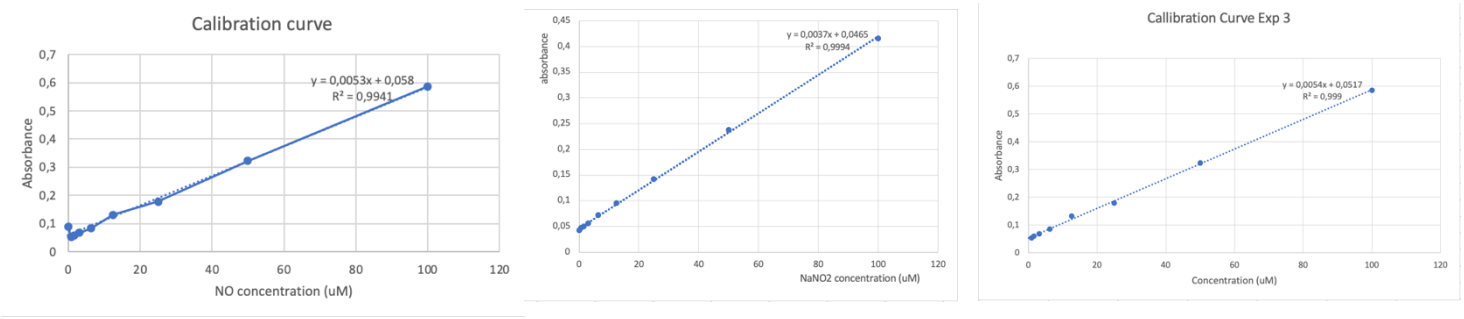


Figure 12 Calibration curves corresponding to the NO assay in figure 4. From left to right: R^2 = 0,9941, R^2 = 0,9994, R^2 = 0,999.

Table 6 Raw data from the NO assay in figure 5

Treatment	*Time (h)*	*Absorbance*		
Control	24	0,048	0,046	0,047
LPS	24	0,178	0,189	0,176
IL-10	24	0,047	0,052	0,049
Pre-stimulation LPS	24	0,193	0,176	0,161
Pre-stimulation IL-10	24	0,158	0,155	0,154
Co-stimulation	24	0,161	0,153	0,161
Control	24	0,050	0,045	0,046
LPS	24	0,128	0,126	0,121
IL-10	24	0,050	0,048	0,049
Pre-stimulation LPS	24	0,115	0,112	0,109
Pre-stimulation IL-10	24	0,130	0,126	0,160
Co-stimulation	24	0,118	0,113	0,110
Control	24	0,048	0,046	0,047
LPS	24	0,178	0,189	0,176
IL-10	24	0,047	0,052	0,049
Pre-stimulation LPS	24	0,193	0,176	0,161
Pre-stimulation IL-10	24	0,158	0,155	0,154
Co-stimulation	24	0,161	0,153	0,161
Control	26	0,063	0,054	0,053

LPS	26	0,198	0,211	0,277
IL-10	26	0,087	0,055	0,052
Pre-stimulation LPS	26	0,294	0,189	0,173
Pre-stimulation IL-10	26	0,224	0,243	0,317
Co-stimulation	26	0,201	0,332	0,195
Control	26	0,050	0,046	0,054
LPS	26	0,126	0,123	0,119
IL-10	26	0,046	0,045	0,047
Pre-stimulation LPS	26	0,112	0,112	0,106
Pre-stimulation IL-10	26	0,121	0,121	0,120
Co-stimulation	26	0,117	0,113	0,107
Control	26	0,063	0,054	0,053
LPS	26	0,198	0,211	0,277
IL-10	26	0,087	0,055	0,052
Pre-stimulation LPS	26	0,294	0,189	0,173
Pre-stimulation IL-10	26	0,224	0,243	0,317
Co-stimulation	26	0,201	0,332	0,195
Treatment	*Time (h)*	*Average absorbance*	*Concentration (μM)*	*Concentration $\pm$ std (μM)*
Control	24	0,047	-2,08	-2,08 $\pm$ -0,044
LPS	24	0,177	22,45	22,45 $\pm$ 0,179
IL-10	24	0,049	-1,64	-1,64 $\pm$ -0,083
Pre-stimulation LPS	24	0,177	22,39	22,39 $\pm$ 2,029
Pre-stimulation IL-10	24	0,156	18,43	18,43 $\pm$ 0,246
Co-stimulation	24	0,158	18,93	19,93 $\pm$ 0,552
Control	24			
LPS	24			
IL-10	24			
Pre-stimulation LPS	24			
Pre-stimulation IL-10	24			
Co-stimulation	24			
Control	24	0,047	-0,870	-0,870 $\pm$ -0,019
LPS	24	0,181	23,94	23,94 $\pm$ 0,926
IL-10	24	0,049	-0,438	-0,438 $\pm$ -0,022
Pre-stimulation LPS	24	0,177	23,14	23,14 $\pm$ 2,097

Pre-stimulation IL-10	24	0,156	19,25	19,25 ± 0,257
Co-stimulation	24	0,158	19,75	19,75 ± 0,576
Control	26			
LPS	26			
IL-10	26			
Pre-stimulation LPS	26			
Pre-stimulation IL-10	26			
Co-stimulation	26			
Control	26	0,057	-0,252	-0,252 ± -0,024
LPS	26	0,204	27,64	27,64 ± 1,24
IL-10	26	0,065	1,26	1,26 ± 0,377
Pre-stimulation LPS	26	0,181	23,21	23,21 ± 1,45
Pre-stimulation IL-10	26	0,234	33,11	33,11 ± 1,905
Co-stimulation	26	0,198	26,42	26,42 ± 0,566
Control	26	0,057	0,919	0,919 ± 0,089
LPS	26	0,229	32,77	32,77 ± 6,07
IL-10	26	0,065	2,40	2,40 ± 0,720
Pre-stimulation LPS	26	0,219	30,92	30,92 ± 9,29
Pre-stimulation IL-10	26	0,261	38,82	38,82 ± 7,299
Co-stimulation	26	0,243	35,36	35,36 ± 11,28

1.4 Raw data used to obtain results in figure 9

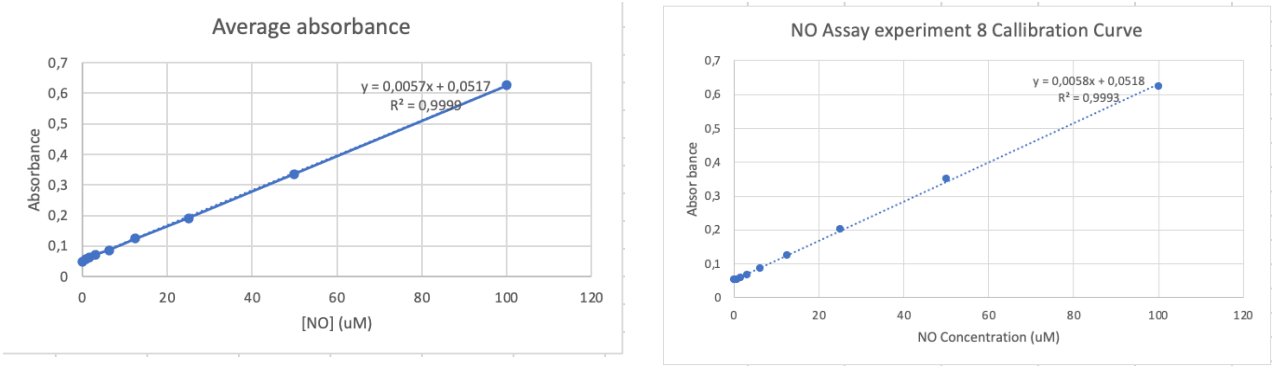


Figure 13 Calibration curves corresponding to the NO assay in figure 10. From left to right: R^2 = 0,9999, R^2 = 0,9993.

Table 7 Raw data used to obtain results from the NO assay from figure 9

Treatment	Absorbance		
Control	0,053	0,050	0,052
IL-10	0,052	0,051	0,048
P1	0,051	0,051	0,051
P2	0,05	0,051	0,051
P3	0,05	0,052	0,05
LPS	0,317	0,341	0,301
IL-10 + LPS	0,307	0,297	0,305
P1 + LPS	0,294	0,323	0,316
P2 + LPS	0,280	0,311	0,275
P3 + LPS	0,229	0,276	0,252
Control	0,054	0,050	0,051
IL-10	0,051	0,050	0,060
P1	0,059	0,052	0,055
P2	0,054	0,056	0,056
P3	0,055	0,054	0,056
LPS	0,263	0,337	0,290
IL-10 + LPS	0,258	0,33	0,321
P1 + LPS	0,229	0,330	0,327
P2 + LPS	0,277	0,321	0,305
P3 + LPS	0,195	0,277	0,249
Treatment	*Average absorbance*	*Concentration (µM)*	*Concentration ± std (µM)*
Control	0,052	-0,0058	-0,0058 ± -0,0002
IL-10	0,050	-0,240	-0,240 ± -0,010
P1	0,051	-0,123	-0,123 ± 0
P2	0,051	-0,181	-0,18 ± -0,002
P3	0,051	-0,182	-0,182 ± -0,004
LPS	0,320	47,01	47,01 ± 2,96
IL-10 + LPS	0,303	44,09	44,09 ± 0,770
P1 + LPS	0,311	45,49	45,49 ± 2,21
P2 + LPS	0,289	41,57	41,57 ± 2,808

P3 + LPS	0,252	35,20	35,20 ± 3,278
Control	0,052	-0,0230	-0,0230 ± -0,0009
IL-10	0,054	0,322	0,322 ± 0,033
P1	0,055	0,609	0,609 ± 0,0387
P2	0,055	0,609	0,609 ± 0,0127
P3	0,055	0,552	0,552 ± 0,010
LPS	0,300	42,22	42,22 ± 5,329
IL-10 + LPS	0,303	43,31	43,31 ± 5,607
P1 + LPS	0,295	41,99	41,99 ± 8,170
P2 + LPS	0,301	42,97	42,97 ± 3,179
P3 + LPS	0,240	32,51	32,51 ± 5,638

Appendix B:

2.1 qPCR Data corresponding to figure 5

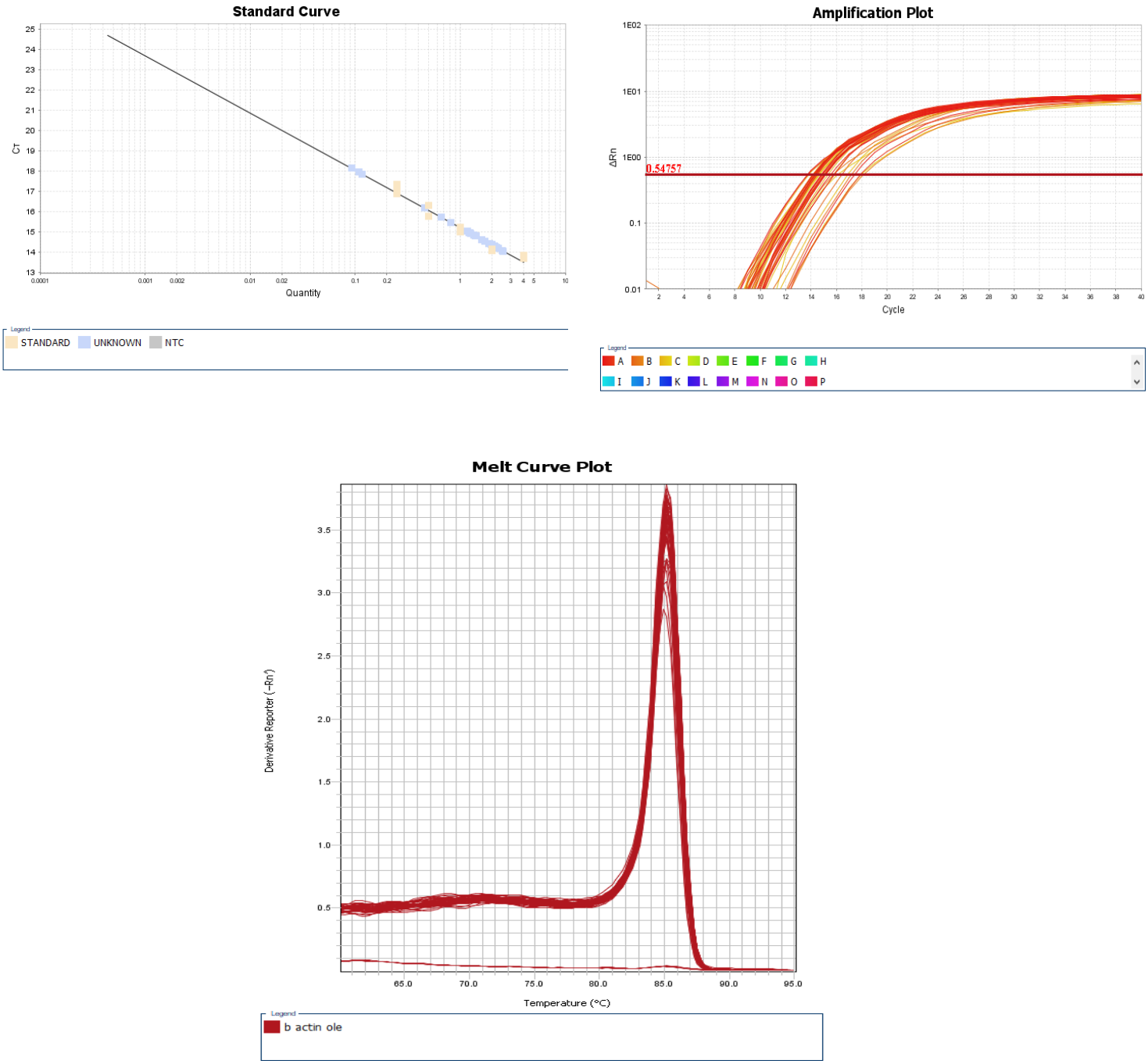


Figure 14 Standard curve, amplification curve and melting curve B-actin qPCR corresponding to sample 1.

Table 8 β-actin Ct, Quantity and Melting Temp. Sample 1

Sample Name	CT	Quantity	Tm1	Tm2
STD4	16,486	4,000	85,912	
STD4	16,981	4,000	85,912	
STD4	17,147	4,000	85,912	
STD2	16,739	2,000	85,912	
STD2	17,499	2,000	86,044	
STD2	17,064	2,000	85,912	
STD1	17,864	1,000	85,912	
STD1	18,489	1,000	86,044	
STD1	18,443	1,000	86,044	
STD0.5	19,558	0,500	85,912	
STD0.5	19,307	0,500	85,912	
STD0.5	19,213	0,500	85,912	
STD0.25	20,805	0,250	85,912	
STD0.25	21,215	0,250	86,044	
STD0.25	21,251	0,250	86,044	
Control 1	17,973	1,441	85,912	
Control 1	18,352	1,127	86,044	
Control 1	18,296	1,170	86,044	
Control 2	17,791	1,622	85,912	
Control 2	18,226	1,224	86,044	
Control 2	17,870	1,542	86,044	
IL-10 1	17,730	1,687	85,912	
IL-10 1	17,781	1,632	86,044	
IL-10 1	17,984	1,431	86,044	
IL-10 2	17,332	2,184	86,044	
IL-10 2	18,111	1,318	86,044	
IL-10 2	17,741	1,675	86,044	
LPS 1	17,399	2,091	86,044	
LPS 1	17,843	1,568	86,176	
LPS 1	17,801	1,612	86,044	
LPS 2	17,600	1,835	86,044	
LPS 2	18,219	1,229	86,176	
LPS 2	18,336	1,140	86,176	
IL-10 pre	18,222	1,227	86,044	
IL-10 pre	17,901	1,510	86,044	
IL-10 pre	18,030	1,389	86,044	
IL-10 pre 2	17,596	1,840	86,044	

IL-10 pre 2	18,178	1,262	86,176	
IL-10 pre 2	17,551	1,895	86,044	
Costim. 1	17,919	1,493	86,044	
Costim. 1	18,112	1,317	86,176	
Costim. 1	18,107	1,322	86,044	
Costim. 2	18,992	0,745	86,044	
Costim. 2	18,187	1,255	86,176	
Costim. 2	17,809	1,603	86,044	
NC 2	Undetermined		85,780	61,371
NC 2	Undetermined		85,780	61,239
NC 2	Undetermined		85,912	61,239
PC 1	18,096	1,331	85,648	
PC 1	17,991	1,425	85,780	
PC 1	18,046	1,375	85,780	

Table 9 β-actin average quantity and corresponding st dev sample 1

Treatment	B-actin quantity	St Dev
Control 1	1,246083	0,17039705
Control 2	1,462347	0,21067231
Cost. 1	1,377079	0,10008503
Costi. 2	1,428832	0,2462503
IL-10 + LPS 1	1,375558	0,142217
IL-10 + LPS 2	1,867315	0,03855021
IL-10 1	1,583540	0,13475189
IL-10 2	1,496513	0,25266024
LPS 1	1,589977	0,03059625
LPS 2	1,184494	0,06351867

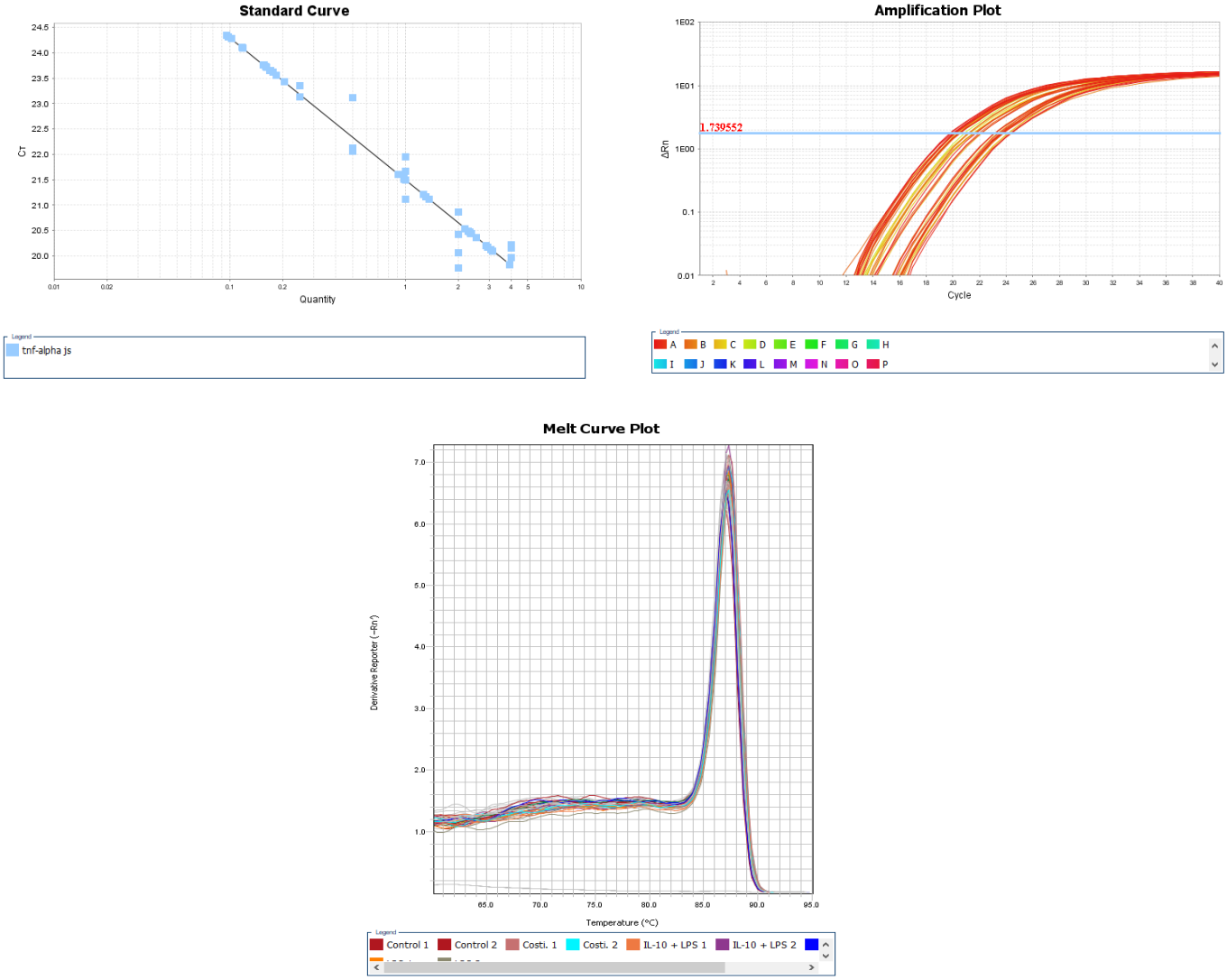


Figure 15 Standard curve, amplification curve and melting curve TNF-α qPCR corresponding to sample 1.

Table 10 TNF-α Ct, Quantity and Melting Temp. Sample 1

Sample Name	CT	Quantity	Tm1	Tm2	Tm3
STD 4	19,974	4,000	87,081		
STD 4	20,216	4,000	87,213		
STD 4	20,161	4,000	87,213		
STD 2	19,765	2,000	86,949		
STD 2	20,066	2,000	87,081		
STD 2	20,419	2,000	87,081		
STD 1	21,665	1,000	87,081		
STD 1	21,943	1,000	87,213		
STD 1	21,109	1,000	87,081		
STD 0.5	22,065	0,500	87,081		
STD 0.5	23,111	0,500	87,213		
STD 0.5	22,119	0,500	87,213		
STD 0.25	23,126	0,250	87,081		
STD 0.25	23,135	0,250	87,213		
STD 0.25	23,358	0,250	87,081		
Control 1	23,759	0,157	87,081		
Control 1	23,560	0,185	87,213		
Control 1	23,765	0,156	87,081		
Control 2	23,613	0,177	87,081		
Control 2	23,653	0,171	87,213		
Control 2	24,095	0,120	87,081		
IL-10 1	24,348	0,097	87,213		
IL-10 1	23,732	0,161	87,213		
IL-10 1	24,107	0,118	87,081		
IL-10 2	24,273	0,103	87,213		
IL-10 2	23,713	0,163	87,213		
IL-10 2	23,658	0,171	87,213		
LPS 1	20,126	3,011	87,213		
LPS 1	20,463	2,290	87,345		
LPS 1	20,435	2,342	87,213		
LPS 2	20,096	3,086	87,213		
LPS 2	21,170	1,289	87,476		
LPS 2	21,603	0,906	87,345		
IL-10 + LPS 1	20,444	2,325	87,213		
IL-10 + LPS 1	21,520	0,970	87,345		
IL-10 + LPS 1	21,511	0,977	87,345		
IL-10 + LPS 2	19,821	3,858	87,345		
IL-10 + LPS 2	20,202	2,831	87,345		
IL-10 + LPS 2	20,356	2,498	87,345		
Costi. 1	20,176	2,891	87,345		

Costi. 1	21,118	1,345	87,345		
Costi. 1	21,496	0,989	87,345		
Costi. 2	20,488	2,243	87,213		
Costi. 2	20,538	2,153	87,345		
Costi. 2	21,204	1,253	87,213		
NC	Undetermined		61,369	87,081	82,466
NC	Undetermined		61,369	87,081	
PC	23,429	0,205	87,213		
PC	24,320	0,100	87,213		

Table 11 TNF-α average quantity and corresponding St Dev from sample 1.

TNF-α	Av quantity	St Dev
Control 1	0,157	0,0005508
Control 2	0,174	0,00396493
Cost. 1	1,167	0,25149113
Costi. 2	2,198	0,06365304
IL-10 + LPS 1	0,973	0,00503212
IL-10 + LPS 2	2,664	0,23602825
IL-10 1	0,125	0,03222393
IL-10 2	0,167	0,00528722
LPS 1	2,316	0,03704877
LPS 2	1,097	0,27073985

Table 12 Relative TNF-α expression and average relative TNF-α expression.

	Relative	Average relative expression	St Dev Average relative expression
Control 1	0,12580186	0,12240311	0,00480657
Control 2	0,11900435		
Cost. 1	0,84737443	1,19300389	0,48879387
Costi. 2	1,53863335		
IL-10 + LPS 1	0,70757555	1,06723788	0,50863935
IL-10 + LPS 2	1,42690022		
IL-10 1	0,0792021	0,09534673	0,02283196
IL-10 2	0,11149136		
LPS 1	1,4567576	1,19161692	0,37496554
LPS 2	0,92647624		

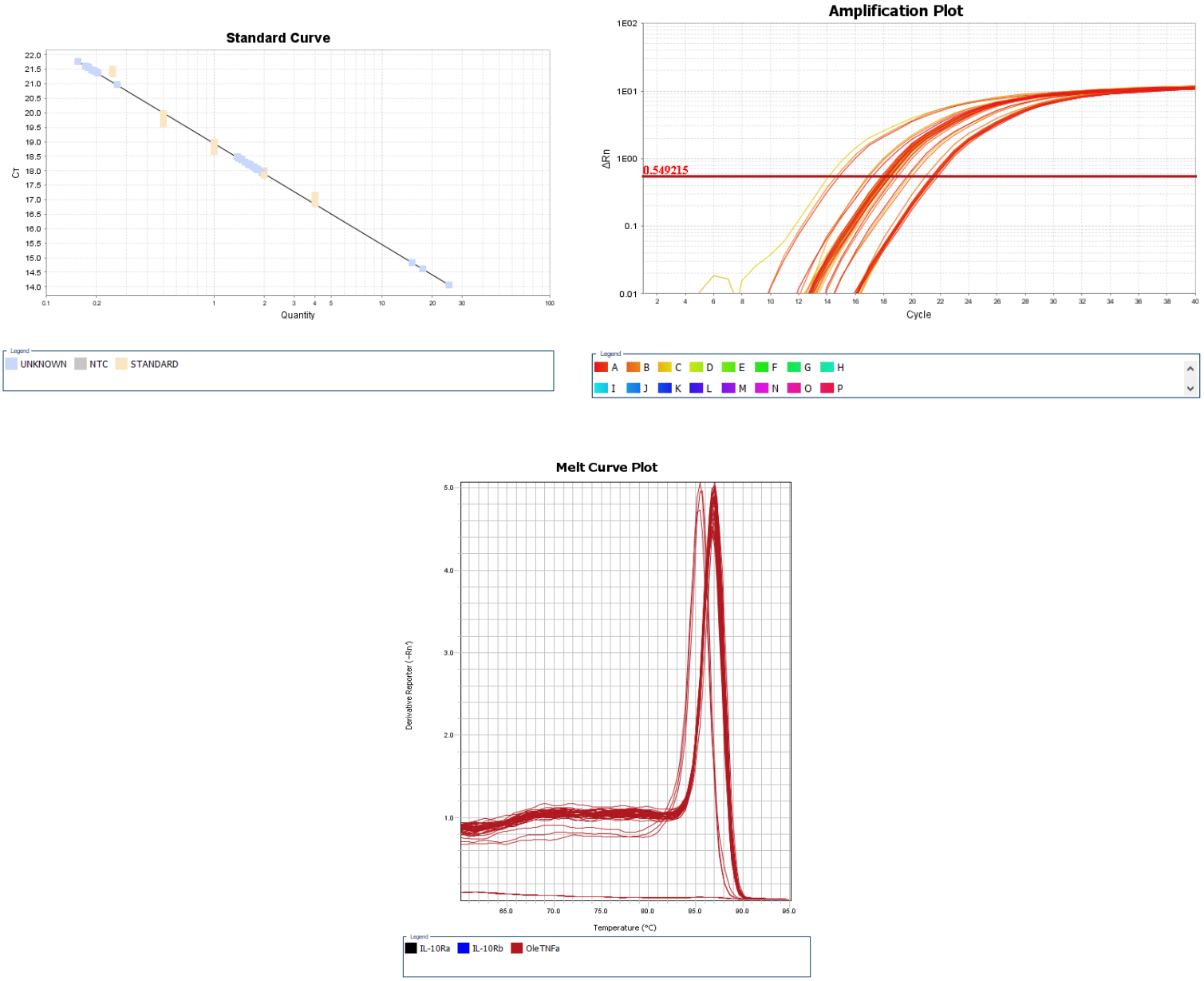


Figure 16 Standard curve, amplification curve and melting curve TNF-alpha qPCR corresponding to sample 2

Table 13 TNF-alpha Ct, Quantity and Melting Temp. Sample 2

Sample Name	Target Name	CT	Quantity	Tm1	Tm2	Tm3
STD4	OleTNFa	17,165	4,000	86,763		
STD4	OleTNFa	16,966	4,000	86,895		
STD4	OleTNFa	16,857	4,000	86,895		
STD2	OleTNFa	17,847	2,000	86,763		
STD2	OleTNFa	17,995	2,000	86,763		
STD2	OleTNFa	17,847	2,000	86,895		
STD1	OleTNFa	18,785	1,000	86,763		
STD1	OleTNFa	18,977	1,000	86,895		
STD1	OleTNFa	18,681	1,000	86,895		
STD0,5	OleTNFa	19,630	0,500	86,763		
STD0,5	OleTNFa	19,961	0,500	86,895		
STD0,5	OleTNFa	19,778	0,500	86,895		
STD0,25	OleTNFa	21,337	0,250	86,763		
STD0,25	OleTNFa	21,502	0,250	86,763		
STD0,25	OleTNFa	22,193	0,250	86,763		
Control 1	OleTNFa	21,567	0,210	86,763		
Control 1	OleTNFa	21,491	0,220	86,895		
Control 1	OleTNFa	23,292	0,073	86,500		
Control 2	OleTNFa	21,778	0,185	86,763		
Control 2	OleTNFa	20,966	0,304	87,158		
Control 2	OleTNFa	21,371	0,237	86,895		
IL-10 1	OleTNFa	21,580	0,209	86,895		
IL-10 1	OleTNFa	21,451	0,226	86,895		
IL-10 1	OleTNFa	21,423	0,230	86,895		
IL-10 2	OleTNFa	21,455	0,225	86,895		
IL-10 2	OleTNFa	21,491	0,220	86,895		
IL-10 2	OleTNFa	21,622	0,203	86,763		
LPS 1	OleTNFa	18,195	1,677	86,895		
LPS 1	OleTNFa	18,311	1,561	86,895		
LPS 1	OleTNFa	18,217	1,655	86,895		
LPS 2	OleTNFa	18,138	1,737	86,895		
LPS 2	OleTNFa	18,197	1,675	87,026		
LPS 2	OleTNFa	18,229	1,643	86,895		
IL-10 pre	OleTNFa	18,426	1,455	87,026		
IL-10 pre	OleTNFa	18,474	1,413	87,026		
IL-10 pre	OleTNFa	18,380	1,496	87,026		
IL-10 pre 2	OleTNFa	17,949	1,951	87,026		
IL-10 pre 2	OleTNFa	18,069	1,812	87,026		

IL-10 pre 2	OleTNFa	17,940	1,962	87,026		
Costim. 1	OleTNFa	18,046	1,838	87,026		
Costim. 1	OleTNFa	18,084	1,795	87,026		
Costim. 1	OleTNFa	17,963	1,935	87,026		
Costim. 2	OleTNFa	18,376	1,500	86,895		
Costim. 2	OleTNFa	18,438	1,444	87,026		
Costim. 2	OleTNFa	18,255	1,617	86,895		
NC	OleTNFa	Undetermined		61,237	92,290	85,579
NC	OleTNFa	Undetermined		85,579	61,368	92,290
NC	OleTNFa	Undetermined		62,158	85,974	83,737
PC	OleTNFa	14,832	13,307	85,316		
PC	OleTNFa	14,620	15,161	85,447		
PC	OleTNFa	14,065	21,335	85,579		
NC	IL-10Ra	Undetermined		61,368		
NC	IL-10Rb	Undetermined		89,921	80,447	61,237

Table 14 TNF-alpha average quantity and corresponding St Dev for sample 2

	Average Quantity	St Dev
Control 1	0,16774548	0,08250649
Control 2	0,24209711	0,06005621
IL-10 1	0,22138794	0,01121699
IL-10 2	0,21626469	0,01277375
LPS 1	1,63106855	0,06146058
LPS 2	1,68473502	0,04795099
IL-10 pre	1,45455253	0,04167659
IL-10 pre 2	1,90843999	0,08365708
CO 1	1,85622493	0,03019426
CO 2	1,52027845	0,08813179

Table 15 Relative TNF-alpha expression and average relative expression for sample 2

Treatment type	Relative TNF-a expression	Average relative expression	St Dev average relative expression
Control 1	0,13461822	0,15008599	0,02187472
Control 2	0,16555375		
IL-10 1	0,13980572	0,14215905	0,0033281
IL-10 2	0,14451237		
LPS 1	1,02584394	1,22408443	0,28035439
LPS 2	1,42232492		
IL-10 pre	1,05742713	1,03972545	0,02503396
IL-10 pre 2	1,02202376		
Costim, 1	1,34794351	1,20597236	0,20077753
Costim, 2	1,0640012		

2.2 qPCR Data corresponding to figure 6

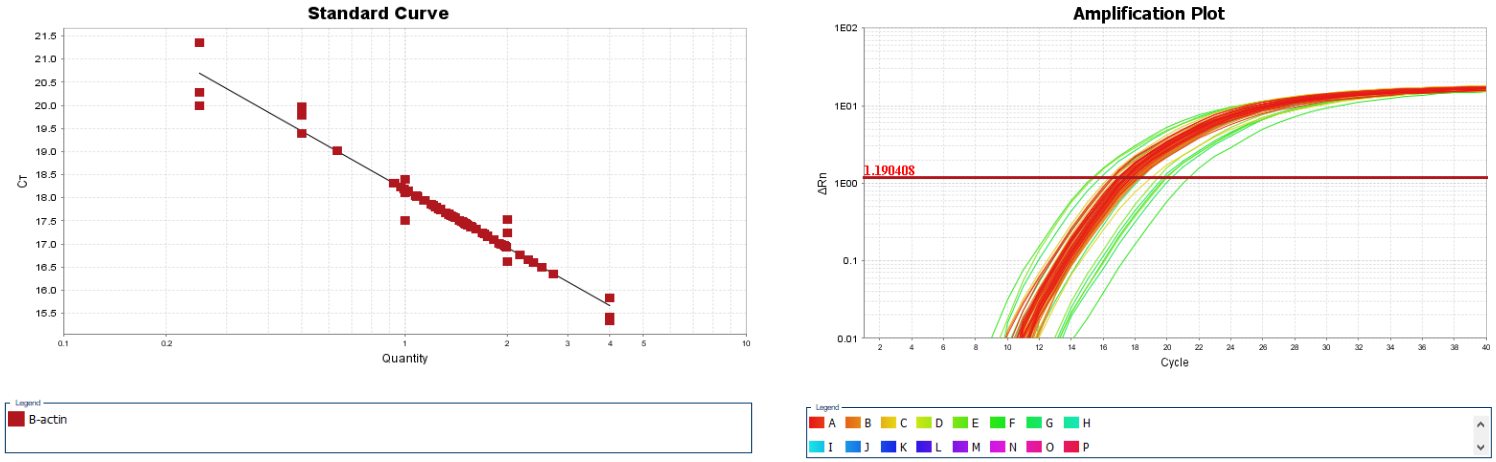


Figure 17 Standard curve, amplification curve and melting curve B-actin qPCR corresponding to sample 1.

Table 16 B-actin Ct, Quantity and Melting Temp. Sample 1

Target Name	CT	Quantity	Tm1
STD 4	15,423	4,000	85,929
STD 4	15,339	4,000	86,061
STD 4	15,838	4,000	86,061
STD 2	17,531	2,000	85,929
STD 2	16,609	2,000	85,929
STD 2	17,247	2,000	86,061
STD 1	17,506	1,000	85,929
STD 1	18,391	1,000	85,929
STD 1	18,102	1,000	85,929
STD 0.5	19,392	0,500	85,797
STD 0.5	19,962	0,500	85,929
STD 0.5	19,778	0,500	86,061
STD 0.25	19,991	0,250	85,797
STD 0.25	21,362	0,250	85,929
STD 0.25	20,273	0,250	85,929
Control 1	16,963	1,957	85,797
Control 1	17,210	1,708	85,797
Control 1	17,441	1,504	85,797
Control 2	17,745	1,272	85,797
Control 2	17,640	1,348	85,797
Control 2	17,950	1,137	85,929
IL-10 1	17,373	1,561	85,797
IL-10 1	17,798	1,235	85,929
IL-10 1	17,411	1,529	85,929
IL-10 2	17,430	1,513	85,797
IL-10 2	17,620	1,363	85,929
IL-10 2	17,787	1,243	85,929
LPS10 1	16,997	1,920	85,797
LPS10 1	18,039	1,082	85,929
LPS10 1	17,568	1,402	85,929
LPS10 2	17,020	1,896	85,797
LPS10 2	17,372	1,562	85,929
LPS10 2	17,427	1,515	85,929
LPS30 1	17,461	1,488	85,797
LPS30 1	18,317	0,929	85,929
LPS30 1	17,449	1,497	85,929
LPS30 2	17,800	1,235	85,929
LPS30 2	18,028	1,089	85,929
LPS30 2	18,050	1,076	85,929

LPS100 1	17,235	1,685	85,929
LPS100 1	17,168	1,748	85,929
LPS100 1	16,773	2,172	85,929
LPS100 2	16,596	2,394	85,929
LPS100 2	16,935	1,987	86,061
LPS100 2	17,450	1,496	85,929
P1 1	17,313	1,614	86,061
P1 1	17,835	1,211	85,929
P1 1	18,033	1,086	86,061
P1 2	17,089	1,825	85,929
P1 2	18,187	0,998	86,061
P1 2	17,516	1,443	86,061
IL-10 + LPS10 1	17,419	1,523	86,061
IL-10 + LPS10 1	18,133	1,028	86,061
IL-10 + LPS10 1	18,139	1,024	86,061
IL-10 + LPS10 2	17,645	1,344	86,061
IL-10 + LPS10 2	17,609	1,371	86,061
IL-10 + LPS10 2	17,567	1,403	86,061
IL-10 + LPS30 1	17,456	1,492	86,061
IL-10 + LPS30 1	16,663	2,308	86,061
IL-10 + LPS30 1	16,354	2,735	86,061
IL-10 + LPS30 2	16,953	1,968	85,929
IL-10 + LPS30 2	17,165	1,751	86,061
IL-10 + LPS30 2	17,681	1,318	86,061
IL-10 + LPS100 1	16,494	2,533	85,929
IL-10 + LPS100 1	17,764	1,259	85,929
IL-10 + LPS100 1	17,860	1,195	85,929
IL-10 + LPS100 2	16,997	1,920	85,929
IL-10 + LPS100 2	17,487	1,466	85,929
IL-10 + LPS100 2	17,357	1,575	85,929
P1 + LPS 100 1	18,225	0,977	85,929
P1 + LPS 100 1	17,680	1,319	85,929
P1 + LPS 100 1	19,010	0,634	85,929
P1 + LPS 100 2	17,933	1,147	85,797
P1 + LPS 100 2	17,584	1,390	85,929
P1 + LPS 100 2	17,736	1,279	85,929
NC	Undetermined		61,369
NC	Undetermined		61,237
NC	Undetermined		61,237
PC	18,308	0,933	85,929
PC	17,476	1,475	85,665
PC	17,491	1,463	85,929

Table 17 B-actin average quantity and corresponding st dev from sample 1.

	Average Quantity	St. dev
Control 1	1,723	0,22663031
Control 2	1,252	0,10685477
IL-10 1	1,442	0,02250675
IL-10 2	1,373	0,13548675
LPS10 1	1,468	0,42294663
LPS10 2	1,658	0,20760038
LPS30 1	1,492	0,0065819
LP30 2	1,082	0,00920361
LPS100 1	1,716	0,04445616
LPS100 2	1,959	0,44937552
P1 1	1,148	0,08826831
P1 2	1,422	0,41405832
IL-10 + LPS10 1	1,026	0,00228301
IL-10 + LPS10 2	1,373	0,02952994
IL-10 + LPS30 1	2,521	0,30179824
IL-10 + LPS30 2	1,859	0,153178
IL-10 + LPS100 1	1,227	0,04548252
IL-10 + LPS100 2	1,520	0,07692862
P1 + LPS100 1	0,977	0,34209888
P1 + LPS100 2	1,272	0,12164316

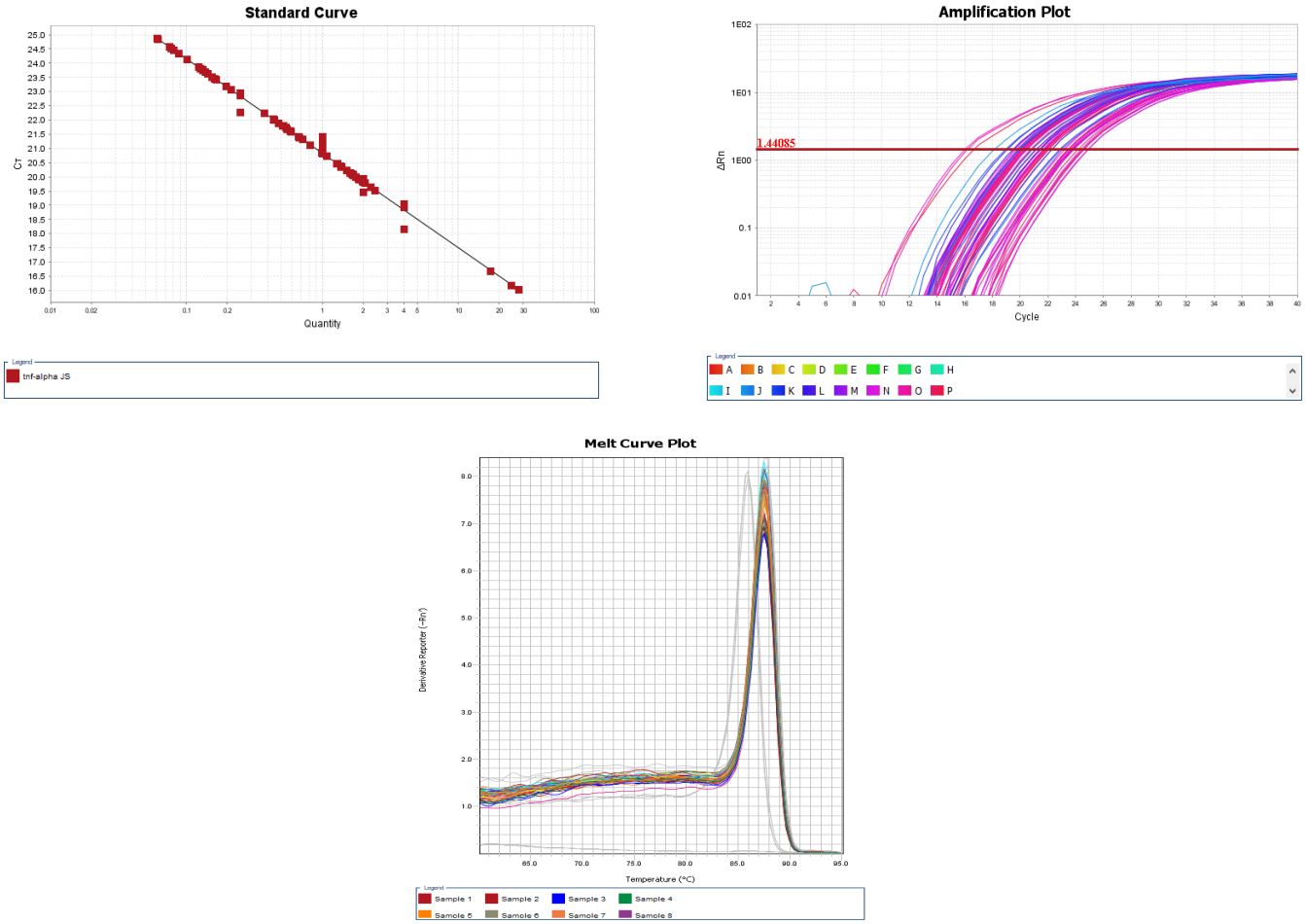


Figure 18 Standard curve, amplification curve and melting curve TNF-alpha qPCR corresponding to sample 1

Table 18 TNF-alpha Ct, Quantity and Melting Temp for sample 1

Sample Name	CT	Quantity	Tm1
STD 4	18,141	4,000	87,688
STD 4	18,926	4,000	87,819
STD 4	19,055	4,000	87,688
STD 2	19,877	2,000	87,688
STD 2	19,920	2,000	87,688
STD 2	19,468	2,000	87,688
STD 1	20,880	1,000	87,688
STD 1	21,136	1,000	87,688
STD 1	21,410	1,000	87,688
Control 1	23,759	0,131	87,425
Control 1	23,696	0,137	87,425
Control 1	24,137	0,101	87,425
Control 2	23,522	0,155	87,425
Control 2	23,855	0,123	87,556
Control 2	23,804	0,127	87,425
IL-10 1	24,346	0,088	87,556
IL-10 1	24,842	0,062	87,556
IL-10 1	24,463	0,081	87,556
IL-10 2	24,865	0,061	87,556
IL-10 2	24,574	0,075	87,556
IL-10 2	24,517	0,078	87,425
LPS10 1	19,907	1,884	87,425
LPS10 1	20,065	1,690	87,556
LPS10 1	20,093	1,657	87,556
LPS10 2	19,778	2,059	87,556
LPS10 2	20,075	1,678	87,556
LPS10 2	19,833	1,982	87,556
LPS30 1	20,451	1,294	87,425
LPS30 1	20,717	1,076	87,556
LPS30 1	20,357	1,381	87,425
LPS30 2	20,001	1,765	87,556
LPS30 2	20,087	1,664	87,556
LPS30 2	20,144	1,599	87,556
LPS100 1	19,812	2,011	87,556
LPS 100 1	20,135	1,610	87,556
LPS 100 1	20,475	1,272	87,556
LPS100 2	19,908	1,883	87,556
LPS100 2	19,938	1,844	87,556
LPS100 2	20,056	1,699	87,556
P1 1	23,750	0,132	87,556
P1 1	23,618	0,145	87,556

P1 1	23,417	0,167	87,556
P1 2	23,058	0,213	87,556
P1 2	23,757	0,132	87,688
P1 2	23,455	0,162	87,688
IL10 + LPS10 1	21,381	0,680	87,556
IL10 + LPS10 1	21,790	0,513	87,688
IL10 + LPS10 1	21,886	0,480	87,688
IL10 + LPS10 2	21,310	0,714	87,556
IL10 + LPS10 2	21,720	0,538	87,688
IL10 + LPS10 2	21,778	0,517	87,688
IL10 + LPS30 1	20,814	1,007	87,556
IL10 + LPS30 1	21,122	0,814	87,688
IL10 + LPS30 1	20,835	0,992	87,688
IL10 + LPS30 2	21,614	0,579	87,425
IL10 + LPS30 2	22,249	0,373	87,556
IL10 + LPS30 2	22,024	0,436	87,688
IL10 + LPS100 1	23,171	0,197	87,425
IL10 + LPS100 1	22,010	0,440	87,556
IL10 + LPS100 1	21,989	0,447	87,556
IL10 + LPS100 2	21,594	0,587	87,556
IL10 + LPS100 2	21,401	0,671	87,556
IL10 + LPS100 2	21,686	0,551	87,556
P1 + LPS 100 1	19,526	2,451	87,425
P1 + LPS 100 1	20,223	1,514	87,556
P1 + LPS 100 1	20,223	1,515	87,556
P1 + LPS 100 2	19,641	2,265	87,425
P1 + LPS 100 2	20,367	1,371	87,556
P1 + LPS 100 2	20,335	1,401	87,556

Table 19 TNF-alpha average quantity and corresponding st dev for sample 1

	Average Quantity	St. dev
Control 1	0,134	0,00413006
Control 2	0,125	0,00313835
IL-10 1	0,084	0,00479961
IL-10 2	0,076	0,00213393
LPS10 1	1,673	0,02318894
LPS10 2	2,021	0,05473046
LPS30 1	1,337	0,06137272
LP30 2	1,676	0,08364326
LPS100 1	1,631	0,3700889
LPS100 2	1,864	0,02728663
P1 1	0,139	0,00893834
P1 2	0,147	0,02163957

IL-10 + LPS10 1	0,496	0,02343974
IL-10 + LPS10 2	0,528	0,01491703
IL-10 + LPS30 1	0,999	0,0103747
IL-10 + LPS30 2	0,405	0,04432101
IL-10 + LPS100 1	0,444	0,004522
IL-10 + LPS100 2	0,569	0,02552173
P1 + LPS100 1	1,514	0,00035572
P1 + LPS100 2	1,386	0,0214138

Table 20 Relative TNF-alpha expression and average relative TNF-alpha expression for sample 1

	Relative expression	Average relative expression	St Dev
Control 1	0,07801534	0,0890239	0,01556846
Control 2	0,10003246		
IL-10 1	0,0584362	0,05703271	0,00198483
IL-10 2	0,05562923		
LPS10 1	1,13948458	1,17927541	0,05627274
LPS10 2	1,21906625		
LPS30 1	0,89610867	1,2222279	0,250152
LP30 2	1,54834712		
LPS100 1	0,95042868	0,95083137	0,00056949
LPS100 2	0,95123406		
P1 1	0,12076688	0,11205462	0,012321
P1 2	0,10334236		
IL-10 + LPS10 1	0,48374787	0,43395707	0,07041483
IL-10 + LPS10 2	0,38416627		
IL-10 + LPS30 1	0,39636134	0,30703519	0,12632625
IL-10 + LPS30 2	0,21770904		
IL-10 + LPS100 1	0,36165162	0,36796534	0,00892895
IL-10 + LPS100 2	0,37427906		
P1 + LPS100 1	1,55056357	1,31999807	0,32606886
P1 + LPS100 2	1,08943257		

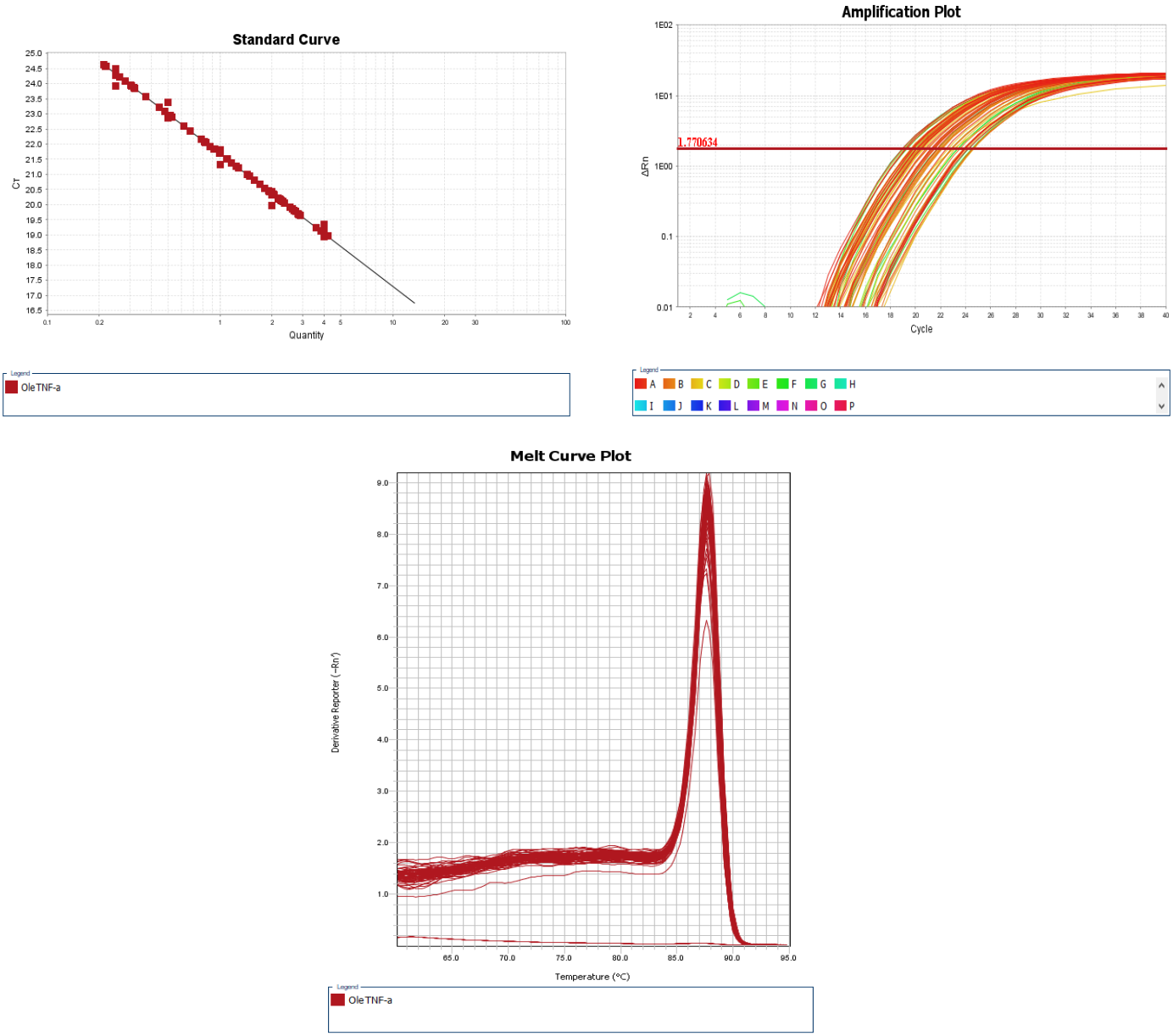


Figure 19 Standard curve, amplification curve and melting curve TNF-alpha qPCR corresponding to sample 2

Table 21 TNF-alpha Ct, Quantity and melting temperature for sample 2

Sample Name	CT	Quantity	Tm1
STD 4	19,111	4,000	87,628
STD 4	19,345	4,000	87,760
STD 4	18,948	4,000	87,760
STD 2	19,962	2,000	87,760
STD 2	20,419	2,000	87,760
STD 2	20,322	2,000	87,760
STD 1	21,333	1,000	87,628
STD 1	21,797	1,000	87,760
STD 1	21,734	1,000	87,760
STD 0,5	22,852	0,500	87,760
STD 0,5	23,392	0,500	87,760
STD 0,5	23,389	0,500	87,760
STD 0,25	23,909	0,250	87,760
STD 0,25	24,491	0,250	87,760
STD 0,25	24,280	0,250	87,760
Control 1	24,230	0,262	87,496
Control 1	23,847	0,320	87,496
Control 1	24,087	0,282	87,628
Control 2	23,927	0,307	87,496
Control 2	23,567	0,371	87,628
Control 2	23,870	0,317	87,628
IL-10 1	23,933	0,306	87,496
IL-10 1	24,581	0,218	87,628
IL-10 1	24,622	0,213	87,760
IL-10 2	23,950	0,303	87,628
IL-10 2	24,568	0,219	87,628
IL-10 2	24,633	0,212	87,628
LPS 10 1	19,683	2,871	87,628
LPS 10 1	19,778	2,731	87,760
LPS 10 1	19,641	2,935	87,628
LPS 10 2	20,174	2,216	87,628
LPS 10 2	20,135	2,263	87,760
LPS 10 2	20,191	2,197	87,628
LPS 30 1	20,439	1,928	87,628
LPS 30 1	20,991	1,442	87,760
LPS 30 1	20,550	1,819	87,628
LPS 30 2	19,810	2,685	87,628
LPS 30 2	20,310	2,064	87,760
LPS 30 2	20,342	2,029	87,760

LPS 100 1	18,957	4,207	87,760
LPS 100 1	20,101	2,304	87,760
LPS 100 1	19,711	2,829	87,760
LPS 100 2	19,138	3,826	87,628
LPS 100 2	19,852	2,627	87,760
LPS 100 2	19,710	2,831	87,628
P1 1	22,446	0,670	87,760
P1 1	22,943	0,516	87,760
P1 1	23,216	0,447	87,760
P1 2	21,706	0,989	87,760
P1 2	22,898	0,528	87,892
P1 2	22,599	0,618	87,760
Il-10 + LPS 10 1	21,272	1,243	87,760
Il-10 + LPS 10 1	21,827	0,928	87,760
Il-10 + LPS 10 1	23,082	0,479	87,628
IL-10 + LPS 10 2	21,216	1,280	87,760
IL-10 + LPS 10 2	21,929	0,879	87,760
IL-10 + LPS 10 2	21,510	1,097	87,760
IL-10 + LPS 30 1	20,462	1,905	87,628
IL-10 + LPS 30 1	20,942	1,479	87,760
IL-10 + LPS 30 1	20,668	1,709	87,628
IL-10 + LPS 30 2	21,390	1,168	87,628
IL-10 + LPS 30 2	22,153	0,782	87,760
IL-10 + LPS 30 2	21,389	1,169	87,628
IL-10 + LPS 100 1	21,501	1,102	87,628
IL-10 + LPS 100 1	22,078	0,813	87,760
IL-10 + LPS 100 1	22,053	0,824	87,760
IL-10 + LPS 100 2	20,821	1,576	87,628
IL-10 + LPS 100 2	22,044	0,828	87,760
IL-10 + LPS 100 2	21,826	0,929	87,760
P1 + LPS 100 1	19,249	3,608	87,496
P1 + LPS 100 1	19,904	2,556	87,628
P1 + LPS 100 1	20,215	2,169	87,628
P1 + LPS 100 2	20,058	2,356	87,496
P1 + LPS 100 2	20,374	1,996	87,628
P1 + LPS 100 2	20,150	2,245	87,628
NC	Undetermined		61,109
NC	Undetermined		61,373
NC	Undetermined		61,241
PC	16,754	13,426	85,913
PC	17,563	8,770	86,045
PC	17,346	9,833	86,177

Table 22 Average TNF -alpha quantity and corresponding st dev for sample 2

	Average quantity tnf-alpha	St Dev
Control 1	0,28816667	0,02969512
Control 2	0,33166778	0,03463817
IL-10 1	0,24561885	0,05245897
IL-10 2	0,24478932	0,05096132
LPS 10 1	2,84573189	0,10424008
LPS 10 2	2,22542596	0,03417419
LPS 30 1	1,72959423	0,25500893
LPS 30 2	2,25957187	0,36925209
LPS 100 1	3,113554	0,98301405
LPS 100 2	3,09468015	0,64178442
P1 1	0,54420251	0,11441224
P1 2	0,71186558	0,24448991
Il-10 + LPS 10 1	0,88363145	0,38396269
IL-10 + LPS 10 2	1,08547368	0,20062018
IL-10 + LPS 30 1	1,69768349	0,21311856
IL-10 + LPS 30 2	1,03967615	0,22348385
IL-10 + LPS 100 1	0,91322186	0,16355245
IL-10 + LPS 100 2	1,11115557	0,40611621
P1 + LPS 100 1	2,77769407	0,74445953
P1 + LPS 100 2	2,19899825	0,18464256

Table 23 Relative TNF-alpha expression and average relative expression for sample 2

Treatment	Relative TNF-a expression	Av relative expression	St dev av. Relative expression
Control 1	0,16725652	0,21604663	0,06899964
Control 2	0,26483675		
IL-10 1	0,17034791	0,17431316	0,00560772
IL-10 2	0,17827841		
LPS 10 1	1,93807848	1,6402987	0,4211242
LPS 10 2	1,34251893		
LPS 30 1	1,32576359	1,65991111	0,47255597
LPS 30 2	1,99405864		
LPS 100 1	1,66659354	1,62313079	0,06146561
LPS 100 2	1,57966804		
P1 1	0,41750293	0,45904203	0,05874515
P1 2	0,50058113		
Il-10 + LPS 10 1	0,74158049	0,76604687	0,0346007
IL-10 + LPS 10 2	0,79051326		
IL-10 + LPS 30 1	0,6734167	0,64633892	0,03829376
IL-10 + LPS 30 2	0,61926114		
IL-10 + LPS 100 1	0,54947352	0,61070863	0,08659952
IL-10 + LPS 100 2	0,67194374		
P1 + LPS 100 1	2,8439073	2,28620917	0,78870427
P1 + LPS 100 2	1,72851103		

2.3 qPCR Data corresponding to figure 7

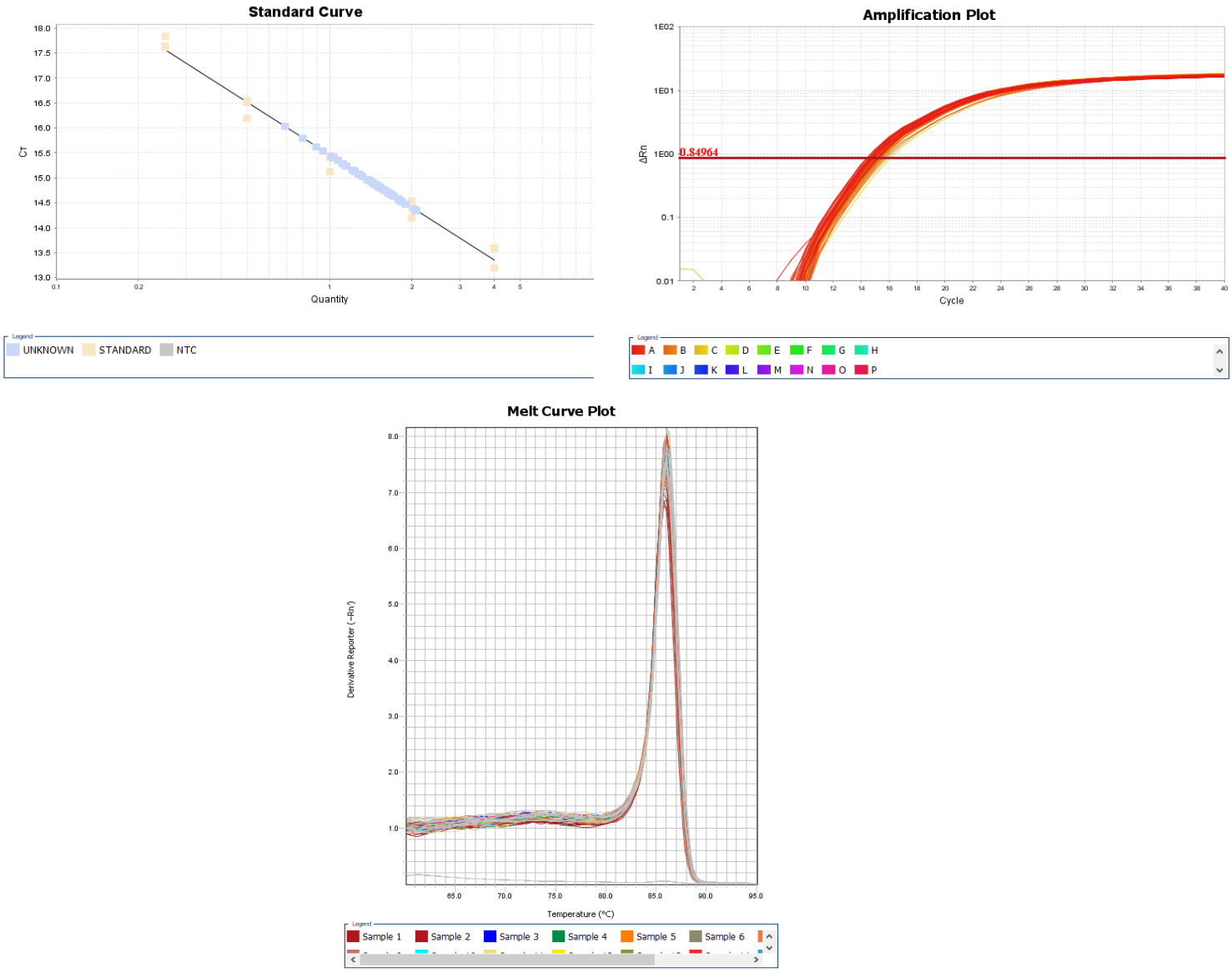


Figure 20 Standard curve, amplification curve and melting curve b-actin qPCR.

Table 24 B-actin Ct, Quantity and Melting temperature.

Sample Name	CT	Quantity	Tm1
STD 4	13,198	4,000	86,042
STD 4	13,588	4,000	86,174
STD 4	13,574	4,000	86,174
STD 2	14,195	2,000	86,042
STD 2	14,540	2,000	86,174
STD 2	14,392	2,000	86,174
STD 1	15,119	1,000	86,042
STD 1	15,418	1,000	86,174
STD 1	15,436	1,000	86,174
STD 0.5	16,193	0,500	86,042
STD 0.5	16,519	0,500	86,174
STD 0.5	16,550	0,500	86,174
STD 0.25	17,618	0,250	86,042
STD 0.25	17,644	0,250	86,042
STD 0.25	17,841	0,250	86,174
Control 1	15,154	1,219	85,778
Control 1	15,394	1,041	85,910
Control 1	15,287	1,117	85,910
Control 2	15,076	1,283	85,778
Control 2	14,960	1,385	85,910
Control 2	15,428	1,018	85,910
IL-10 1	14,864	1,475	85,778
IL-10 1	15,064	1,293	85,910
IL-10 1	14,944	1,400	85,910
IL-10 2	14,689	1,655	85,910
IL-10 2	14,785	1,553	85,910
IL-10 2	14,752	1,588	85,910
P1 1	14,542	1,823	85,778
P1 1	14,815	1,523	85,910
P1 1	14,806	1,532	85,910
P1 2	14,341	2,081	85,910
P1 2	14,696	1,648	85,910
P1 2	14,561	1,800	85,910
P2 1	14,673	1,672	85,910
P2 1	15,083	1,277	86,042
P2 1	14,836	1,502	85,910
P2 2	14,476	1,903	85,910
P2 2	14,754	1,586	86,042
P2 2	14,859	1,480	85,910
P3 1	14,519	1,851	85,910
P3 1	15,025	1,327	86,042
P3 1	14,846	1,492	86,042

P3 2	14,575	1,784	86,042
P3 2	14,885	1,454	86,042
P3 2	14,901	1,440	86,042
LPS 30 1	14,372	2,038	86,042
LPS 30 1	14,656	1,691	86,042
LPS 30 1	14,555	1,807	86,042
LPS30 2	14,857	1,482	86,042
LPS30 2	15,241	1,151	86,042
LPS30 2	14,959	1,386	86,042
LPS30 + IL-10 1	14,846	1,492	86,042
LPS30 + IL-10 1	15,411	1,029	86,174
LPS30 + IL-10 1	15,342	1,077	86,042
LPS30 + IL-10 2	15,126	1,242	86,042
LPS30 + IL-10 2	15,235	1,156	86,174
LPS30 + IL-10 2	15,534	0,949	86,042
LPS30 + P1 1	14,346	2,073	86,042
LPS30 + P1 1	14,948	1,395	86,174
LPS30 + P1 1	14,644	1,705	86,042
LPS30 + P1 2	14,827	1,511	86,042
LPS30 + P1 2	15,056	1,300	86,042
LPS30 + P1 2	14,960	1,385	86,042
LPS30 + P2 1	14,680	1,665	85,910
LPS30 + P2 1	14,818	1,520	86,042
LPS30 + P2 1	14,738	1,602	86,042
LPS30 + P2 2	14,626	1,724	85,910
LPS30 + P2 2	14,970	1,376	86,042
LPS30 + P2 2	14,925	1,417	86,042
LPS30 + P3 1	14,962	1,383	85,910
LPS30 + P3 1	15,277	1,124	86,042
LPS30 + P3 1	15,138	1,232	86,042
LPS30 + P3 2	14,802	1,536	85,910
LPS30 + P3 2	14,705	1,637	85,910
LPS30 + P3 2	15,156	1,217	86,042
NC	Undetermined		61,238
NC	Undetermined		85,910
NC	Undetermined		85,778
PC	15,621	0,896	85,778
PC	15,800	0,797	85,910
PC	16,028	0,686	85,910

Table 25 B-actin average quantity and corresponding st dev.

Sample	Av Quantity	St Dev
Control 1	1,125	0,08959991
Control 2	1,334	0,07213433
IL-10 1	1,389	0,091333
IL-10 2	1,570	0,02428451
P1 1	1,528	0,00655097
P1 2	1,724	0,10792704
P2 1	1,484	0,19804508
P2 2	1,533	0,07482844
P3 1	1,410	0,11694065
P3 2	1,447	0,01035986
LPS30 1	1,749	0,08226492
LPS30 2	1,434	0,06830765
LPS30 + IL-10 1	1,053	0,03399152
LPS30 + IL-10 2	1,199	0,06075949
LPS30 + P1 1	1,724	0,33927007
LPS30 + P1 2	1,399	0,10627138
LPS30 + P2 1	1,596	0,07238338
LPS30 + P2 2	1,396	0,02888585
LPS30 + P3 1	1,246	0,13023292
LPS30 + P3 2	1,587	0,07157749

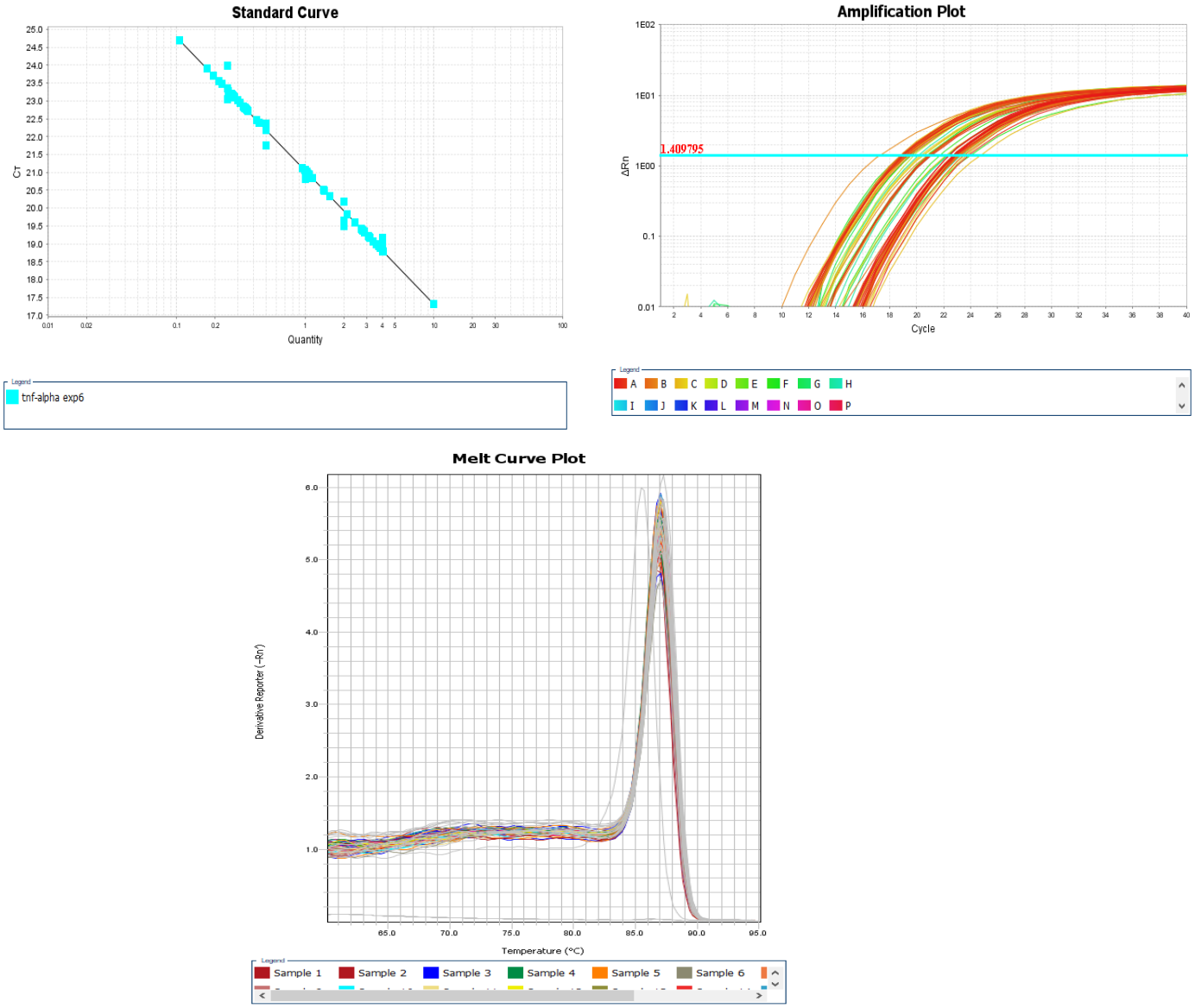


Figure 21 Standard curve, amplification curve and melting curve TNF-alpha qPCR

Table 26 TNF-alpha Ct, Quantity and Melting Temperature.

Sample Name	CT	Quantity	Tm1
STD 4	18,877	4,000	87,165
STD 4	19,155	4,000	87,297
STD 4	18,988	4,000	87,297
STD 2	19,650	2,000	87,033
STD 2	19,488	2,000	87,297
STD 2	20,173	2,000	87,033
STD 1	20,819	1,000	87,033
STD 1	20,873	1,000	87,165
STD 1	21,077	1,000	87,165
STD 0.5	21,748	0,500	87,033
STD 0.5	22,283	0,500	87,165
STD 0.5	22,355	0,500	87,165
STD 0.25	23,976	0,250	86,902
STD 0.25	23,045	0,250	87,033
STD 0.25	23,357	0,250	87,033
Control 1	22,455	0,423	86,770
Control 1	22,800	0,342	86,902
Control 1	23,719	0,194	87,033
Control 2	22,388	0,441	86,902
Control 2	22,715	0,360	86,902
Control 2	22,801	0,342	86,902
IL-10 1	22,950	0,312	86,770
IL-10 1	23,562	0,214	86,902
IL-10 1	23,101	0,284	87,033
IL-10 2	22,832	0,336	86,902
IL-10 2	23,285	0,254	86,902
IL-10 2	22,857	0,330	87,033
P1 1	22,817	0,339	86,902
P1 1	23,146	0,277	87,033
P1 1	22,808	0,340	87,033
P1 2	22,935	0,315	86,902
P1 2	22,723	0,359	87,033
P1 2	24,704	0,106	87,033
P2 1	22,742	0,355	86,902
P2 1	23,188	0,269	87,033
P2 1	23,010	0,301	87,033
P2 2	23,911	0,173	87,033
P2 2	22,846	0,333	87,033
P2 2	22,723	0,359	87,033
P3 1	22,942	0,314	87,033
P3 1	22,723	0,359	87,033
P3 1	22,787	0,345	86,902

P3 2	22,760	0,351	87,033
P3 2	23,177	0,271	87,165
P3 2	23,121	0,281	87,033
LPS30 1	18,793	4,029	87,033
LPS30 1	20,333	1,562	87,165
LPS30 1	20,489	1,419	87,033
LPS30 2	19,172	3,192	87,033
LPS30 2	19,422	2,737	87,165
LPS30 2	19,830	2,129	87,165
LPS30 + IL-10 1	20,937	1,077	87,165
LPS30 + IL-10 1	21,009	1,030	87,165
LPS30 + IL-10 1	20,970	1,055	87,033
LPS30 + IL-10 2	21,132	0,955	87,033
LPS30 + IL-10 2	21,079	0,987	87,165
LPS30 + IL-10 2	22,187	0,499	87,165
LPS30 + P1 1	19,388	2,794	87,033
LPS30 + P1 1	18,993	3,563	87,165
LPS30 + P1 1	18,786	4,047	87,033
LPS30 + P1 2	19,189	3,158	87,033
LPS30 + P1 2	18,926	3,712	87,165
LPS30 + P1 2	20,524	1,389	87,033
LPS30 + P2 1	19,071	3,395	87,033
LPS30 + P2 1	18,994	3,561	87,033
LPS30 + P2 1	19,205	3,126	87,033
LPS30 + P2 2	18,883	3,814	86,902
LPS30 + P2 2	19,329	2,897	87,033
LPS30 + P2 2	19,207	3,123	87,033
LPS30 + P3 1	19,077	3,384	86,902
LPS30 + P3 1	19,602	2,449	87,033
LPS30 + P3 1	19,377	2,813	86,902
LPS30 + P3 2	18,977	3,597	86,902
LPS30 + P3 2	20,851	1,135	87,033
LPS30 + P3 2	19,341	2,877	86,902
NC	Undetermined		86,770
NC	Undetermined		86,244
NC	Undetermined		61,365
PC	22,719	0,360	86,902
PC	17,320	9,975	85,585
PC	23,472	0,226	86,902

Table 27 Average TNF-alpha quantity and corresponding st dev.

Sample	Average Quantity	St dev
Control 1	0,383	0,05728273
Control 2	0,351	0,01314004
IL-10 1	0,270	0,05052383
IL-10 2	0,333	0,00358935
P1 1	0,340	0,00139413
P1 2	0,337	0,03110718
P2 1	0,285	0,02202877
P2 2	0,346	0,01845771
P3 1	0,352	0,00981558
P3 2	0,276	0,00679902
LPS30 1	2,337	1,46717585
LPS30 2	2,686	0,53342469
LPS30 + IL-10 1	1,054	0,02334593
LPS30 + IL-10 2	0,971	0,02222745
LPS30 + P1 1	3,468	0,63165717
LPS30 + P1 2	3,435	0,3923644
LPS30 + P2 1	3,361	0,21956119
LPS30 + P2 2	3,010	0,16001405
LPS30 + P3 1	2,882	0,47142057
LPS30 + P3 2	3,237	0,50944649

Table 28 Relative TNF alpha expression and average relative TNF-alpha expression.

Sample	Relative expression	Average Relative Expression	St dev average relative expression
Control 1	0,340017313	0,301650227	0,05425925
Control 2	0,26328314		
IL-10 1	0,194405341	0,203217653	0,01246249
IL-10 2	0,212029964		
P1 1	0,222219181	0,208798598	0,01897957
P1 2	0,195378014		
P2 1	0,192110757	0,208794642	0,02359458
P2 2	0,225478528		
P3 1	0,249566966	0,220186409	0,04155038
P3 2	0,190805852		
LPS30 1	1,33583536	1,604468647	0,37990484
LPS30 2	1,873101934		
LPS30 + IL-10 1	1,000879139	0,905448791	0,13495889
LPS30 + IL-10 2	0,810018444		
LPS30 + P1 1	2,010992258	2,233343017	0,31445146
LPS30 + P1 2	2,455693776		
LPS30 + P2 1	2,106103211	2,13107916	0,03532133
LPS30 + P2 2	2,15605511		
LPS30 + P3 1	2,312582262	2,176454017	0,19251441
LPS30 + P3 2	2,040325772		

Appendix C - Protocols

3.1 Protocol NO assay

Protocol NO assay

1. Seed RAW 264.7 cells in a 96-well flat bottom culture plate (100.000 cells in 200 µl/well) in DMEM supplemented with 10% FBS.
2. After 24h, remove supernatant. Add fresh medium containing the following treatment.
    a. Control
    b. LPS 25 ng/ml
    e. LPS 100 ng/ml
3. After 24h incubation, collect 100 ul of the supernatant to measure NO_2^- (one of the end products of NO synthesis)

NO assay:

Materials:

- 100 mM NaNO2 stock solution
- 96 well plate
- 1,5 ml tubes for the standard curve
- Medium of the cells
- Griess solutions:
    - Griess A and Griess B

Calibration curve of Sodium Nitrite ($NaNO_2$):

1. Prepare stock-solution: 100 mM $NaNO_2$-solution in MQ (0.69 g/100 ml)
   (Store stock-solution in vials at -20°C)

2. Dilute stock-solution 100x in culture medium (= 1 mM solution).
   Pipet 100 ul 100 mM NaNO2 in 10 ml medium 1 mM NaNO2

3. Make the standard curve:

[NaNO2] (uM)	**V NaNO2**	**V medium**
100	100 ul 1 mM	900 ul
50	500 ul 100 uM	500 ul
25	500 ul 50 uM	500 ul
12.5	500 ul 25 uM	500 ul
6.3	500 ul 12,5 uM	500 ul
3.1	500 ul 6.3 uM	500 ul
1.6	500 ul 3,1 uM	500 ul
0.8	500 ul 1,6 uM	500 ul
0	-	500 ul

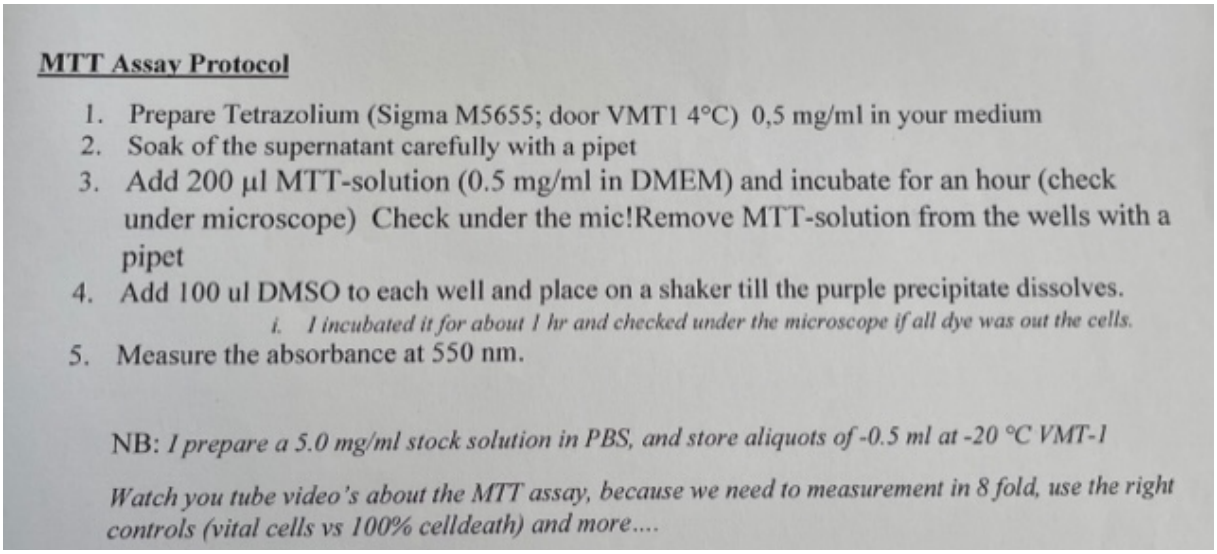
The reaction:

1. Pipet 100 ul of the standard curve samples in triplo in a 96 well plate
2. Pipet 100 ul of your experimental samples in empty wells
3. Make fresh Griess reagent bij mixing equal volume of Griess A and Griess B
4. Pipet 100 ul of this fresh prepared Griess to all the standards and samples
5. Remove the bubbles out of the wells (they disturb the readout)
6. Measure the plate at 550 nM

Griess reagents:
Griess A: 2gr Sulfanilamide en 5 ml fosforzuur in total volume of 100ml MQ
Griess B: 200mg N-(1-Naphthyl)ethylenediamine dihydrochloride in100ml MQ
1:1 mengen vlak voor gebruik

$NaNo_2$ stock
0.69 g $NaNo_2$/100 mL MiliQ water
$NaNo_2$ = #1772 in weighing room

3.2 Protocol MTT assay



MTT Assay Protocol

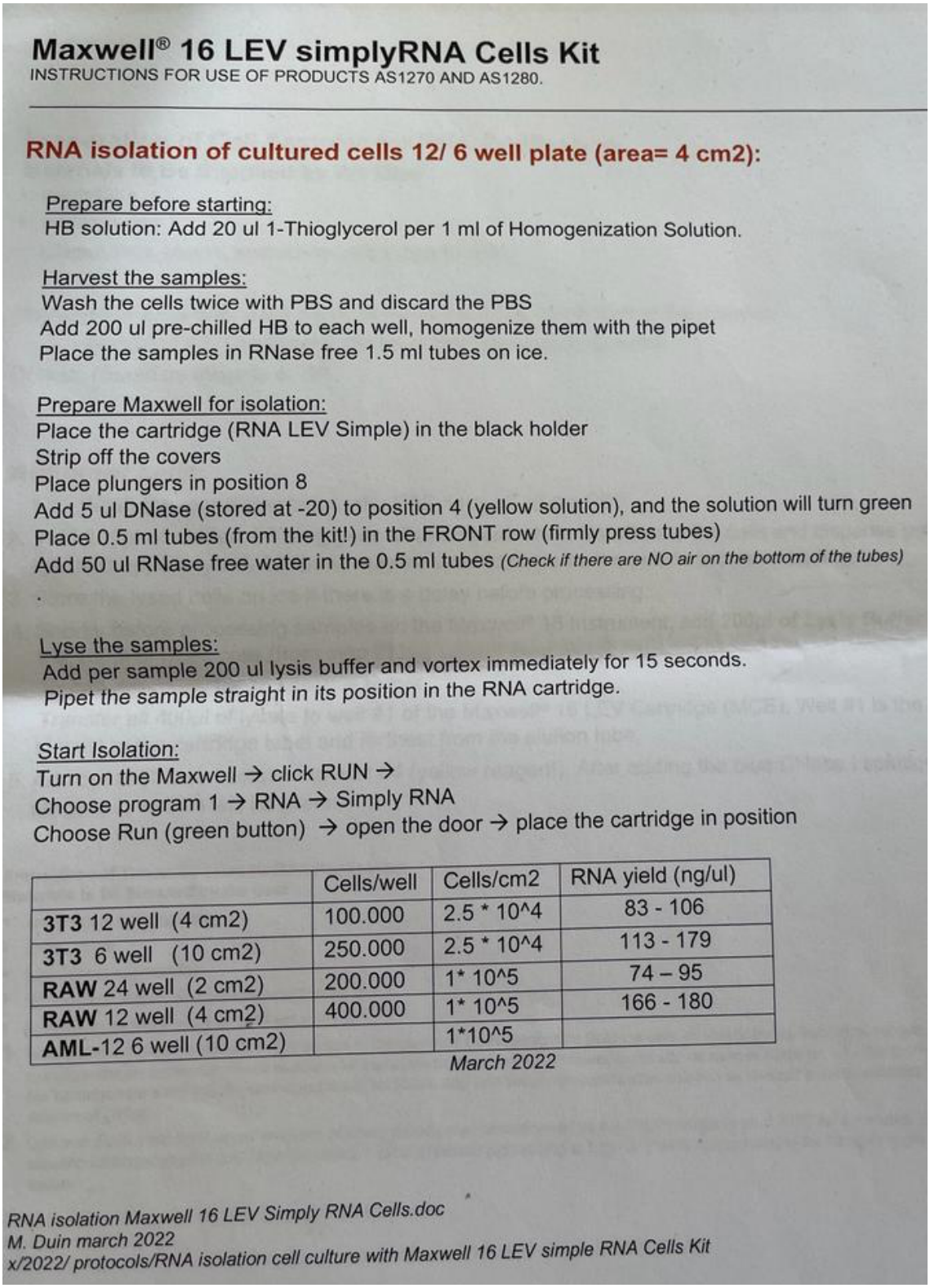
1. Prepare Tetrazolium (Sigma M5655; door VMT1 4°C) 0,5 mg/ml in your medium
2. Soak of the supernatant carefully with a pipet
3. Add 200 µl MTT-solution (0.5 mg/ml in DMEM) and incubate for an hour (check under microscope) Check under the mic!Remove MTT-solution from the wells with a pipet
4. Add 100 ul DMSO to each well and place on a shaker till the purple precipitate dissolves.
   i. *I incubated it for about 1 hr and checked under the microscope if all dye was out the cells.*
5. Measure the absorbance at 550 nm.

NB: *I prepare a 5.0 mg/ml stock solution in PBS, and store aliquots of -0.5 ml at -20 °C VMT-1*

Watch you tube video's about the MTT assay, because we need to measurement in 8 fold, use the right controls (vital cells vs 100% celldeath) and more....

Figure 22 The protocol for the MTT assay

3.3 Maxwell® 16 LEV simplyRNA Cells kit



Maxwell® 16 LEV simplyRNA Cells Kit
INSTRUCTIONS FOR USE OF PRODUCTS AS1270 AND AS1280.

RNA isolation of cultured cells 12/ 6 well plate (area= 4 cm2):

Prepare before starting:
HB solution: Add 20 ul 1-Thioglycerol per 1 ml of Homogenization Solution.

Harvest the samples:
Wash the cells twice with PBS and discard the PBS
Add 200 ul pre-chilled HB to each well, homogenize them with the pipet
Place the samples in RNase free 1.5 ml tubes on ice.

Prepare Maxwell for isolation:
Place the cartridge (RNA LEV Simple) in the black holder
Strip off the covers
Place plungers in position 8
Add 5 ul DNase (stored at -20) to position 4 (yellow solution), and the solution will turn green
Place 0.5 ml tubes (from the kit!) in the FRONT row (firmly press tubes)
Add 50 ul RNase free water in the 0.5 ml tubes *(Check if there are NO air on the bottom of the tubes)*
.

Lyse the samples:
Add per sample 200 ul lysis buffer and vortex immediately for 15 seconds.
Pipet the sample straight in its position in the RNA cartridge.

Start Isolation:
Turn on the Maxwell → click RUN →
Choose program 1 → RNA → Simply RNA
Choose Run (green button) → open the door → place the cartridge in position

	Cells/well	Cells/cm2	RNA yield (ng/ul)
3T3 12 well (4 cm2)	100.000	2.5 * 10^4	83 - 106
3T3 6 well (10 cm2)	250.000	2.5 * 10^4	113 - 179
RAW 24 well (2 cm2)	200.000	1* 10^5	74 – 95
RAW 12 well (4 cm2)	400.000	1* 10^5	166 - 180
AML-12 6 well (10 cm2)		1*10^5	

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RNA isolation Maxwell 16 LEV Simply RNA Cells.doc
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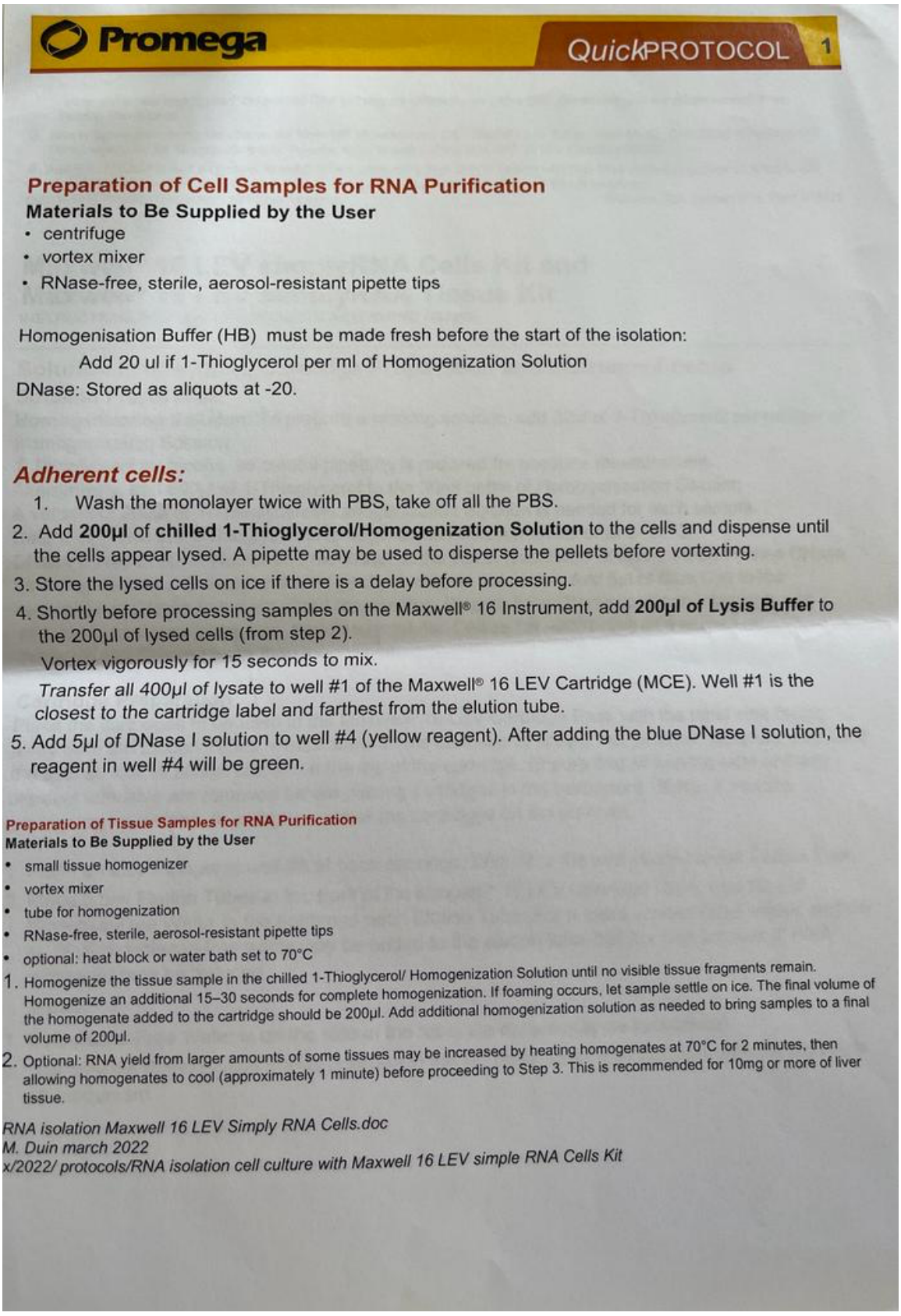
x/2022/ protocols/RNA isolation cell culture with Maxwell 16 LEV simple RNA Cells Kit

Figure 23 RNA isolation protocol.


Promega QuickPROTOCOL 1

Preparation of Cell Samples for RNA Purification

Materials to Be Supplied by the User

- centrifuge
- vortex mixer
- RNase-free, sterile, aerosol-resistant pipette tips

Homogenisation Buffer (HB) must be made fresh before the start of the isolation:
Add 20 ul if 1-Thioglycerol per ml of Homogenization Solution
DNase: Stored as aliquots at -20.

Adherent cells:

1. Wash the monolayer twice with PBS, take off all the PBS.
2. Add **200µl** of **chilled 1-Thioglycerol/Homogenization Solution** to the cells and dispense until the cells appear lysed. A pipette may be used to disperse the pellets before vortexting.
3. Store the lysed cells on ice if there is a delay before processing.
4. Shortly before processing samples on the Maxwell® 16 Instrument, add **200µl of Lysis Buffer** to the 200µl of lysed cells (from step 2).
   Vortex vigorously for 15 seconds to mix.
   Transfer all 400µl of lysate to well #1 of the Maxwell® 16 LEV Cartridge (MCE). Well #1 is the closest to the cartridge label and farthest from the elution tube.
5. Add 5µl of DNase I solution to well #4 (yellow reagent). After adding the blue DNase I solution, the reagent in well #4 will be green.

Preparation of Tissue Samples for RNA Purification

Materials to Be Supplied by the User

- small tissue homogenizer
- vortex mixer
- tube for homogenization
- RNase-free, sterile, aerosol-resistant pipette tips
- optional: heat block or water bath set to 70°C

1. Homogenize the tissue sample in the chilled 1-Thioglycerol/ Homogenization Solution until no visible tissue fragments remain. Homogenize an additional 15–30 seconds for complete homogenization. If foaming occurs, let sample settle on ice. The final volume of the homogenate added to the cartridge should be 200µl. Add additional homogenization solution as needed to bring samples to a final volume of 200µl.
2. Optional: RNA yield from larger amounts of some tissues may be increased by heating homogenates at 70°C for 2 minutes, then allowing homogenates to cool (approximately 1 minute) before proceeding to Step 3. This is recommended for 10mg or more of liver tissue.

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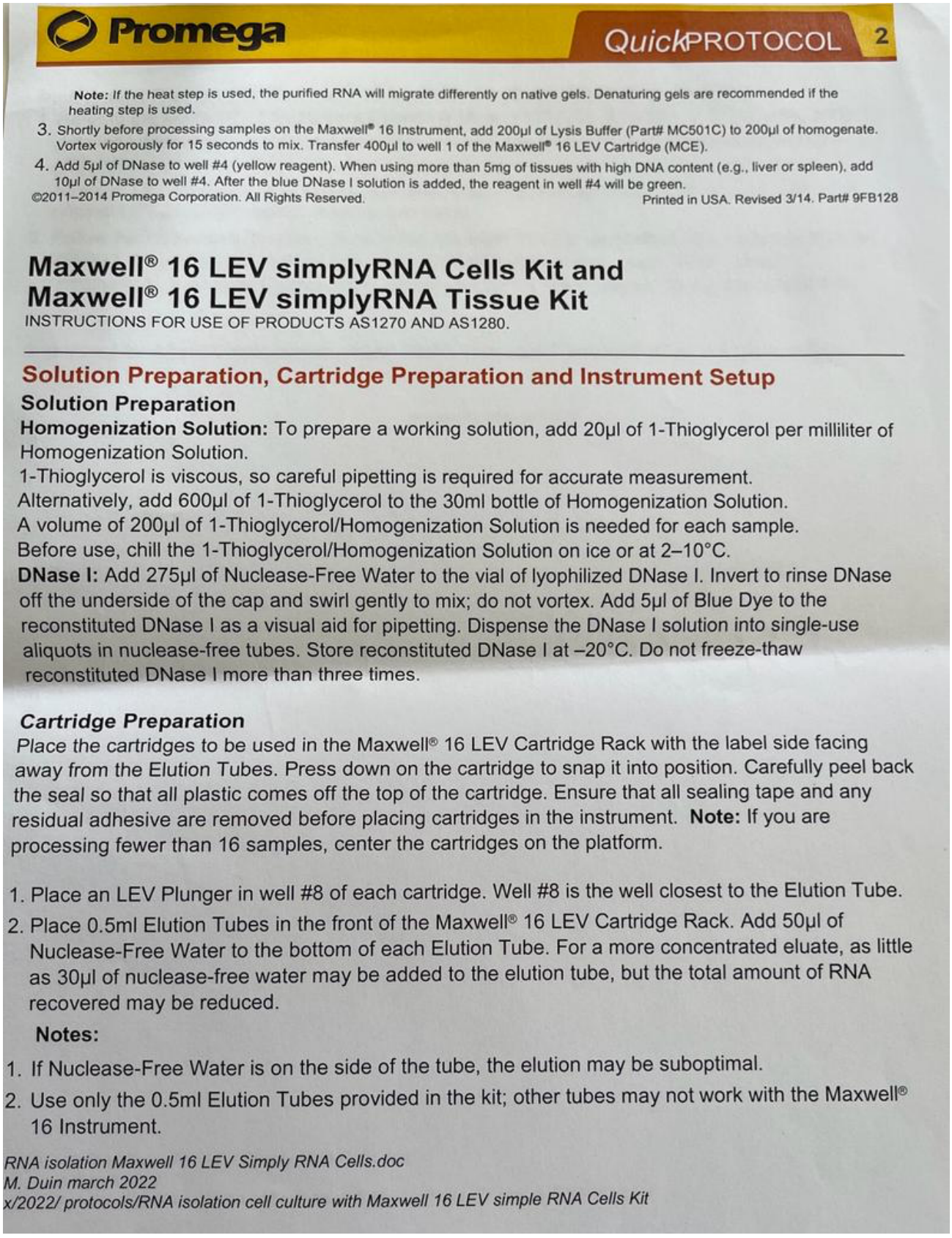
Figure 24 RNA isolation protocol.



Promega QuickPROTOCOL 2

Note: If the heat step is used, the purified RNA will migrate differently on native gels. Denaturing gels are recommended if the heating step is used.

3. Shortly before processing samples on the Maxwell® 16 Instrument, add 200µl of Lysis Buffer (Part# MC501C) to 200µl of homogenate. Vortex vigorously for 15 seconds to mix. Transfer 400µl to well 1 of the Maxwell® 16 LEV Cartridge (MCE).
4. Add 5µl of DNase to well #4 (yellow reagent). When using more than 5mg of tissues with high DNA content (e.g., liver or spleen), add 10µl of DNase to well #4. After the blue DNase I solution is added, the reagent in well #4 will be green.

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Maxwell® 16 LEV simplyRNA Cells Kit and Maxwell® 16 LEV simplyRNA Tissue Kit

INSTRUCTIONS FOR USE OF PRODUCTS AS1270 AND AS1280.

Solution Preparation, Cartridge Preparation and Instrument Setup

Solution Preparation

Homogenization Solution: To prepare a working solution, add 20µl of 1-Thioglycerol per milliliter of Homogenization Solution.
1-Thioglycerol is viscous, so careful pipetting is required for accurate measurement.
Alternatively, add 600µl of 1-Thioglycerol to the 30ml bottle of Homogenization Solution.
A volume of 200µl of 1-Thioglycerol/Homogenization Solution is needed for each sample.
Before use, chill the 1-Thioglycerol/Homogenization Solution on ice or at 2–10°C.
DNase I: Add 275µl of Nuclease-Free Water to the vial of lyophilized DNase I. Invert to rinse DNase off the underside of the cap and swirl gently to mix; do not vortex. Add 5µl of Blue Dye to the reconstituted DNase I as a visual aid for pipetting. Dispense the DNase I solution into single-use aliquots in nuclease-free tubes. Store reconstituted DNase I at –20°C. Do not freeze-thaw reconstituted DNase I more than three times.

Cartridge Preparation

Place the cartridges to be used in the Maxwell® 16 LEV Cartridge Rack with the label side facing away from the Elution Tubes. Press down on the cartridge to snap it into position. Carefully peel back the seal so that all plastic comes off the top of the cartridge. Ensure that all sealing tape and any residual adhesive are removed before placing cartridges in the instrument. **Note:** If you are processing fewer than 16 samples, center the cartridges on the platform.

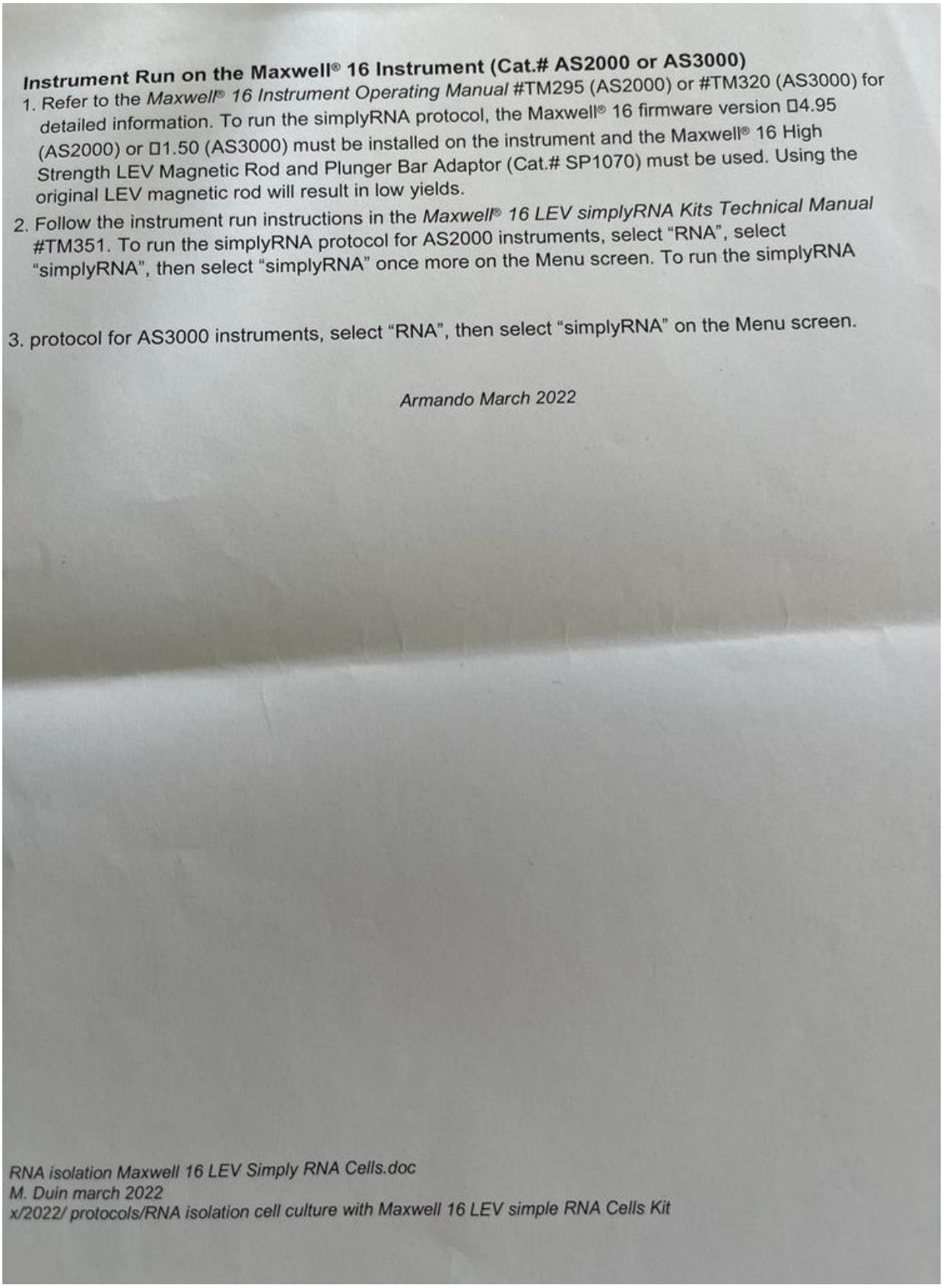
1. Place an LEV Plunger in well #8 of each cartridge. Well #8 is the well closest to the Elution Tube.
2. Place 0.5ml Elution Tubes in the front of the Maxwell® 16 LEV Cartridge Rack. Add 50µl of Nuclease-Free Water to the bottom of each Elution Tube. For a more concentrated eluate, as little as 30µl of nuclease-free water may be added to the elution tube, but the total amount of RNA recovered may be reduced.

Notes:

1. If Nuclease-Free Water is on the side of the tube, the elution may be suboptimal.
2. Use only the 0.5ml Elution Tubes provided in the kit; other tubes may not work with the Maxwell® 16 Instrument.

RNA isolation Maxwell 16 LEV Simply RNA Cells.doc
M. Duin march 2022
x/2022/ protocols/RNA isolation cell culture with Maxwell 16 LEV simple RNA Cells Kit

Figure 25 RNA isolation protocol.



Instrument Run on the Maxwell® 16 Instrument (Cat.# AS2000 or AS3000)

1. Refer to the *Maxwell® 16 Instrument Operating Manual* #TM295 (AS2000) or #TM320 (AS3000) for detailed information. To run the simplyRNA protocol, the Maxwell® 16 firmware version □4.95 (AS2000) or □1.50 (AS3000) must be installed on the instrument and the Maxwell® 16 High Strength LEV Magnetic Rod and Plunger Bar Adaptor (Cat.# SP1070) must be used. Using the original LEV magnetic rod will result in low yields.
2. Follow the instrument run instructions in the *Maxwell® 16 LEV simplyRNA Kits Technical Manual* #TM351. To run the simplyRNA protocol for AS2000 instruments, select "RNA", select "simplyRNA", then select "simplyRNA" once more on the Menu screen. To run the simplyRNA
3. protocol for AS3000 instruments, select "RNA", then select "simplyRNA" on the Menu screen.

Armando March 2022

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Figure 26 RNA isolation protocol.

3.4 PCR machine protocol

RNA conversion to cDNA

Use this protocol after RNA isolation with the Maxwell and on-column DNA digestion.

Precaution:
tubes, tips en water must be RNase free.
You yourself are the source of Rnase

RT mix:

RT buffer		2.0 ul	
dNTP(=A,G,C,T)mix (10 mM)	0.1 ul		
Rnasin	0.25 ul		(=10 units)
Rev Transcriptase		0.5 ul	(=100 units)
Random Hexamers		0.5 ul	(=0.5 ug)
RNA		0.5 ug	(preferably in 5 ul)
H_20		1.5 ul	(to get total vol. of 10 ul)
		--------	+
Total volume		10 ul	

NB: *Add extra samples for the standard curve !!*

Converting RNA tot cDNA:

10 min 20 °C
30 min 42 °C
10 min 20 °C
5 min 99 °C
5 min 20 °C

Place the tubes in the PCR machine
Start the file ***MLVCDNA***

After the reaction is completed:

Spin the tubes (condensed water from the lids)
Store the samples at -20.

Costs: € 3/sample

Basic materials Promega:

M-MLV Rev Transcriptase
cat nr: M1705 (= 5* 10.000 units)
per 10.000 U → 50 reactions
price: € 175,=/10.000 units

RNasin
cat. nr.: N 2515 (= 10.000 U)
per 10.000 U → 500 reactions
price: € 230,=/10.000 units

Random Hexamers
cat nr.: C1181 (=20 ug)
per 20 µg → 40 reactions
price: € 27,=/ 20 ug

dNTP's dATP, dCTP, dGTP, dTTP set 100 uM/nucl.
cat nr: U1245
each nucl. 400 ul → 5.000 reactions
price: €240,=

Rnase free filtertips

P10	771288	10 boxes steriel/pkg	€45	Greiner
P20	774288	10 boxes steriel/ pkg	€45	Greiner
P100	772288	10 boxes steriel/ pkg	€45	Greiner
P200	739288	10 boxes steriel/ pkg	€45	Greiner
P1000	740288	10 boxes steriel/ pkg	€45	Greiner

Thin wall 0,5 ml tube Rnase and Dnase free

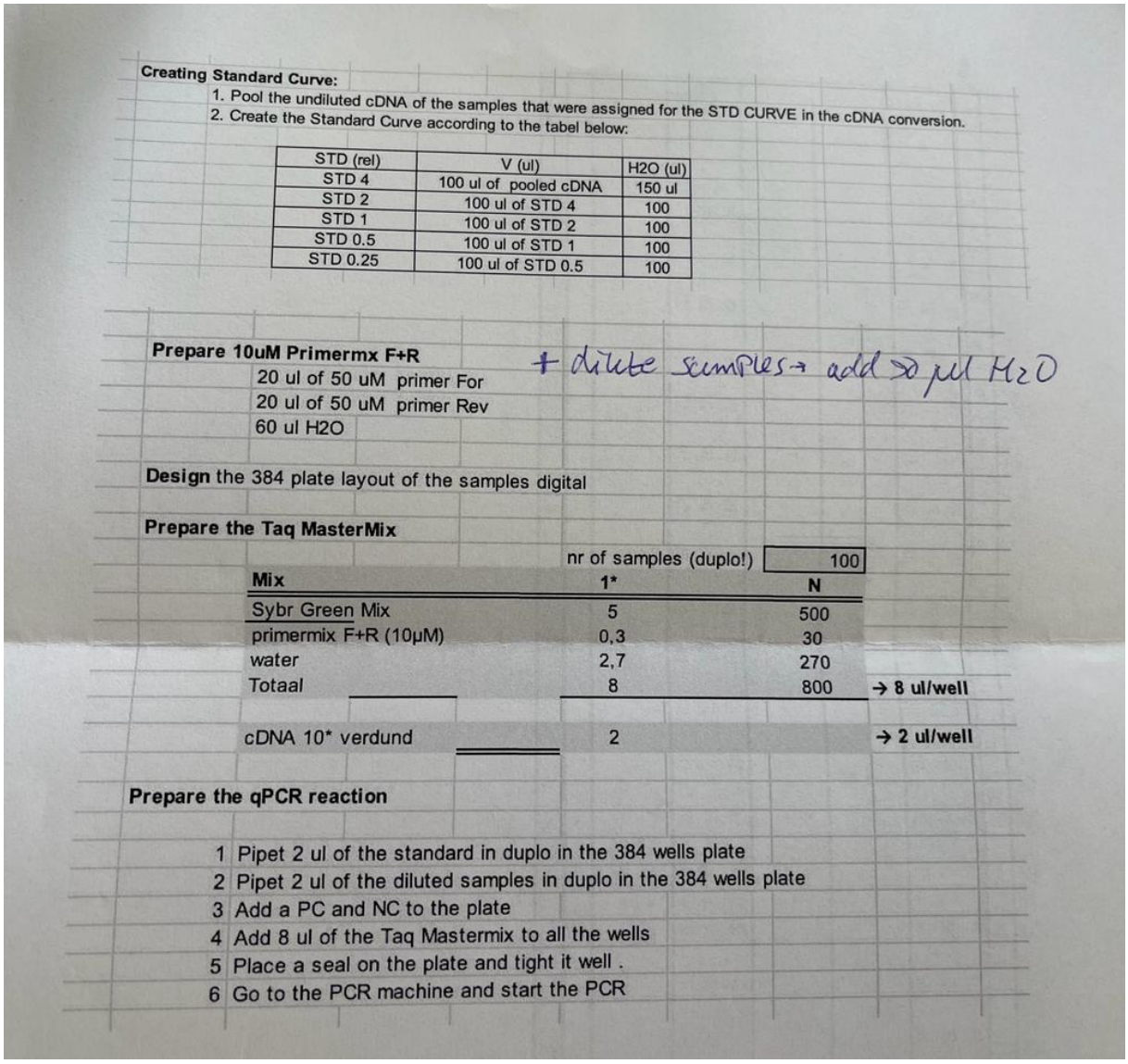
B79801	1000/bag	€38	Biozym

Waste

medibin

P113758-2022/protocols/RNA and DNA/RNA conversion to cDNA (10 ul version)

3.5 PCR protocol



Creating Standard Curve:

1. Pool the undiluted cDNA of the samples that were assigned for the STD CURVE in the cDNA conversion.
2. Create the Standard Curve according to the tabel below:

STD (rel)	V (ul)	H2O (ul)
STD 4	100 ul of pooled cDNA	150 ul
STD 2	100 ul of STD 4	100
STD 1	100 ul of STD 2	100
STD 0.5	100 ul of STD 1	100
STD 0.25	100 ul of STD 0.5	100

Prepare 10uM Primermx F+R

20 ul of 50 uM primer For
20 ul of 50 uM primer Rev
60 ul H2O

+ dilute samples → add 20 µl H_2O

Design the 384 plate layout of the samples digital

Prepare the Taq MasterMix

nr of samples (duplo!) 100

Mix	1*	N	
Sybr Green Mix	5	500	
primermix F+R (10µM)	0,3	30	
water	2,7	270	
Totaal	8	800	→ 8 ul/well
cDNA 10* verdund	2		→ 2 ul/well

Prepare the qPCR reaction

1. Pipet 2 ul of the standard in duplo in the 384 wells plate
2. Pipet 2 ul of the diluted samples in duplo in the 384 wells plate
3. Add a PC and NC to the plate
4. Add 8 ul of the Taq Mastermix to all the wells
5. Place a seal on the plate and tight it well .
6. Go to the PCR machine and start the PCR

Figure 27 PCR protocol