Steroid receptors in the brain of female Japanese quail – A protocol for qPCR

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Summary

Based on a variety of studies investigating brain function in birds, it is now clear that birds possess a 'social behavior network', that is homologous to the social behavior network in mammals. Because the social behavior network contains steroid receptors, hormones play an important role in social behavior. The sensitivity to a certain hormone is determined by the expression of the receptor for this hormone. As a proxy for expression of a receptor itself, one can quantify amounts of RNA, which reflects gene expression. A very reliable method for determining the expression of genes, is the qPCR.

For isolating RNA, different protocols were tested. A mixed protocol between isolation with trizol and isolation with the RNeasy Plus Microkit (50) gave the best results. After isolation of RNA, cDNA was prepared, as cDNA was necessary as starting material for the qPCR.

For the qPCR, primers were tested for reference genes and for the different steroid receptors. The primers for reference genes gave better results than the primers for the steroid receptors, but no optimal results were reached yet. Amplification of cDNA started in the latest cycles of the qPCR, so it did not become clear if the right primers were used.

For further investigation, I would suggest to continue using the mixed protocol for RNA isolation, and to keep investigating ways to start amplification of cDNA in earlier cycles of the qPCR, so it can be determined if the right primers are used.

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1. Introduction

1.1. The social brain

Based on a variety of studies investigating brain function in birds, it is now clear that birds possess a 'social behavior network', that is homologous to the social behavior network in mammals. In total, the social behavior network is comprised of six nodes: the extended medial amygdala (the medial amygdala and the medial bed nucleus of stria terminalis (BSTm)), the lateral septum, the preoptic area, the anterior hypothalamus, the ventromedial hypothalamus, and midbrain areas such as the periaqueductal grey (PAG) and various regions of the tegmentum. [3][4] Each of the nodes has been implicated in the control of multiple forms of social behavior, including aggression, appetitive and consummatory sexual behavior, various forms of communication, social recognition, affiliation, bonding, parental behavior and responses to social stressors. The nodes are bidirectionally connected and each node of the network responds to a variety of stimuli, but each social context and behavioral response is associated with a distinct pattern of response across the nodes. [3]

In birds, the posterior and medial archistriatum is thought to be homologous to the amygdala in mammals, because of its connections to the hypothalamus via the hypothalamic-occipitomesencephalic tract. A discrete nucleus was found within the posterior and medial archistriatium: the nucleus taenia (TnA). [1] Yamamota et al. *(2005)* showed that the TnA is comparable to the mammalian medial amygdala. [2]

1.2.Hormones

Steroid hormones are important regulators of physiology and behavior. They respond to stimuli in the environment and affect the brain. Because the social behavior network contains sex steroid-receptors, it is clear that hormones play an important role in social behavior. It has been shown that the social environment of a female bird has an influence on her endocrine system. These changes in the endocrine system, in turn, have an influence on the social behavior and endocrine system of the offspring of these female birds. This can be described as maternal effects. [5]

In a study by V.C. Goerlich-Jansson (2011), it was investigated if female Japanese quail housed under different social conditions, in groups or in pairs, showed differences in their levels of hormones. It was found that there were indeed differences in their plasma levels of testosterone and corticosterone. [5] If you want to say something about the sensitivity to a certain hormone, not only the concentration of this hormone is important, but also the expression of the receptor for this hormone. When a bird has a high expression of a certain receptor, this bird is more sensitive to changes in the concentration of the hormone binding to this receptor. With lower expression of the receptor, the bird becomes less sensitive to changes in the concentration of the hormone binding to this receptor. So, because significant differences were shown in the levels of hormones in the Japanese quail, the question raised if there would also be a difference in the expression of the steroid-

receptors in the brain. As a proxy for expression of the receptor itself, one can quantify amounts of RNA, which reflects gene expression.
A very reliable method for determining the expression of certain genes, is the qPCR.

1.3. The qPCR

Real time qPCR is the most sensitive and reliable method for detection and quantification of nucleic acid levels. [7] The starting material for this type of PCR is always RNA. [8] So, before starting with the actual qPCR, RNA needs to be isolated from the tissue of interest. After isolation of RNA, the amount of RNA that is isolated can be measured with the BioDrop. The BioDrop not only measures the amount of RNA, but also the A260/A280-value. This value gives information about the purity of the sample. A A260/A280-value between 1.7 and 2.2 represents a pure sample. Low A260/A280-values can be caused by residual reagent associated with the extraction protocol, but very low amounts of RNA can also give a low A260/A280-value.[9] It is very important that other forms of nucleic acids, for example DNA, are no longer present in the sample, as this could disturb the measurements. To make sure of this, a DNase treatment is done after RNA isolation, to destroy DNA.

After isolation of RNA and the DNase treatment, cDNA needs to be prepared. cDNA is double-stranded DNA synthesized out of a single-strand of RNA. cDNA is made out of RNA with reverse transcription. The produced cDNA is the starting material for the actual qPCR.

The qPCR is used to determine the expression of certain genes of interest. The difference between PCR and qPCR is that PCR is just used for amplification of DNA. If you want to determine the amount of DNA, you need to prepare an electrophoretic gel. In qPCR, a fluorescent dye is added, for example, EvaGreen, which lights up in double-stranded DNA. This ensures that the outcome of the qPCR also gives information about the amount of DNA, so you can say something about the expression of the genes of interest. Because for most genes, there is not a standard amount of expression, it is necessary to include so called reference genes. Reference genes are genes that are always present in a standard amount. The expression of the genes of interest can then be showed as an amount of expression compared with the amount of expression of the reference genes. These values are thus no absolute values, but relative values.

For the qPCR to amplify the cDNA, a few chemicals need to be add to the cDNA before starting. The 5x HOT FIREPol EvaGreen qPCR Mix Plus contains all of these chemicals: DNA polymerase, qPCR buffer, $MgCl_2$, dNTPs, EvaGreen dye and No ROX dye. Only water and the primers must be add.
First, the primers need to bind at the 3'-side of the cDNA template. After that, DNA polymerase can add nucleotides to the template. DNA polymerase always adds nucleotides from the 3'-side to the 5'-side. dNTP stands for deoxyribonucleotide triphosphate, referring to the four deoxyribonucleotides dATP, dCTP, dGTP and dTTP. The used primers need to have the right nucleotide-sequence to be able to bind to the cDNA. When the primers don't bind to the cDNA-strand, DNA polymerase can not add nucleotides to the complementary strand, and the DNA can not be amplified.

The outcome of the qPCR is presented with several graphics. The first graphic is the amplification graphic. In this graphic, you can see which primers gave a result, so which primers gave an actual amplification of the cDNA, and in which quantity. The second graphic is the log scale of the amplification graphic. This graphic also shows which primers gave an amplification of the cDNA. The most optimal result of the q-PCR would be reached when this graphic shows a line that becomes horizontal at a certain point. This would mean that the optimal amount of amplification would be reached.
The last graphics are the melt curve and the melt peak. The melt curve shows the strength of the binding of the primers to the cDNA, with a peak at the most optimal temperature. The melt peak is used to analyze whether the qPCR has produced single, specific products. When the melt peak shows one peak, that means that the qPCR produced a pure, single amplicon. When the melt peak shows more peaks, that means that the qPCR produced more than one amplicon. This can be caused by the fact that EvaGreen binds to double-stranded DNA, but is not sequence specific, so it can bind to different nucleotide-sequences, producing different qPCR products. [6]

1.4. Goal of the study

The purpose of this study is to set up a protocol for a qPCR (reverse transcriptase) to measure the gene expression of steroid-receptors in the brain of the Japanese quail.

There are four things that are very important to ensure that the optimal results. We need to set up protocols for:

- How to cut the brain in the right way to get samples from the regions of interest.
- How to extract the RNA in the best way to get high quality RNA for the qPCR.
- Test different primer sequences from the literature.
- How to execute the qPCR in the right way and find the suitable protocol for each primer.

2. Materials & methods

2.1 Brain tissue preparation

For this investigation, brain tissue from Japanese quail *(Coturnix c. japonica)* was used. Before use, the brains were stored by -80 °C. The brains were cut with the Cryostat, in slices of 200 µm. After cutting, micropunches of the hippocampus, hypothalamus (POM) and amygdala (BnST and TnA) were taken by hand. These micropunches were used to isolate RNA from.

2.2 RNA isolation

Three different protocols for isolating RNA from the brain tissue were tested: The RNeasy Plus Micro kit (50), Trizol, and a combination of these two protocols.

2.2.1. The RNeasy Plus Micro kit (50) *(Appendix 1)*

The first protocol that was tested was the protocol for RNA isolation with the RNeasy Plus Micro kit (50) The protocol *'Purification of total RNA from animal and human tissue'* (p24-30) was used in two different ways.

2.2.1.1. The protocol without adding extra carrier RNA

2.2.1.1.a. Materials

The following chemicals & materials were necessary following this protocol:

- The RNeasy Plus Micro Kit (containing gDNA eliminator spin columns, RNeasy minelute spin columns, 1.5 ml collection tubes, 2 ml collection tubes, buffer RLT plus, buffer RW1, buffer RPE, RNase-free water, carrier RNA, poly-A and the handbook).
- Fresh or frozen tissue (no more than 5 mg)
- TissueLyzer
- TissueLyzer adapter set 2x24
- 2 ml microcentrifuge tubes
- Stainless steel beads (3.2 mm)
- Microcentrifuge
- BioDrop
- Pipettes (1000 µl, 200 µl, 2-20 µl)

2.2.1.1.b. Chemicals

- B-Mercaptoethanol
- Ethanol (96-100%)

2.2.1.1.c. Preparations

Before starting with the actual protocol, a few things needed to be prepared.

- β-Mercaptoethanol (β-ME) must be added to Buffer RLT Plus before use, 10 µl of β-ME per 1 ml of Buffer RLT Plus. In this case, 4 tubes were used, and 350 µl of Buffer

RLT Plus must be added to each tube. To be sure there was enough buffer, some extra buffer was made, so 1750 µl of Buffer RLT Plus was prepared (the amount that would have been necessary for 5 tubes). So, 10 µl of β-ME per 1 ml of Buffer RLT Plus meant in this case 17,5 µl of β-ME.

- Before Buffer RPE can be used, 4 volumes of ethanol (96-100%) must be added. Also, 80% ethanol must be prepared by mixing 24 ml ethanol (96-100%) and 6 ml RNase-free water. 70% ethanol was prepared by diluting ethanol (96-100%) with distilled water.

2.2.1.1.d. Notes to the protocol

Step 1: One of the things that is very important before starting the protocol, is to be sure that the samples don't weigh more than 5 mg.
Step 2b: A stainless steel bead with a mean diameter of 3.2 mm was used, instead of a stainless steel bead with a mean diameter of 5 mm. The Tissuelyzer II Qiagen was used for the disruption and homogenization. After homogenization, the tubes were spun down in the Eppendorf Centrifuge 5415C, to get rid of the foam that formed in the tubes.
Step 10: The amount of RNA was measured with the BioDrop. 1.5 µl of each sample was used.

2.2.1.2. The protocol with adding extra carrier RNA

2.2.1.2.a. Materials

The same materials were used as in 1.1.a.

2.2.1.2.b. Chemicals

- Carrier RNA (included in the RNeasy Plus Micro Kit)

Furthermore, the same chemicals were used as in 1.1.b.

2.2.1.2.c. Preparations

- The carrier RNA needed to be prepared for use. The carrier RNA (310 µg) must be dissolved in 1 ml RNase-free water, so the concentration of this stock solution is 310 µg/ml.
- In our test, a working solution of 4 ng/ml was needed. To get to this concentration, 5 µl of stock solution were added to 34 µl Buffer RLT Plus and mixed by pipetting. After that, 6 µl of this diluted solution were added to 54 µl Buffer RLT Plus. This gave the right working solution.

Furthermore, the same preparations were done as in 1.1.c.

2.2.1.2.d. Notes to the protocol

Step 2: Before starting the disruption and homogenization, 5 µl of the carrier RNA solution must be added to the lysate.
Furthermore, the same notes are valid as in 1.1.d.

2.2.2. Trizol *(Appendix 2)*

The second protocol that was tested is the protocol for RNA isolation with trizol. The full specification of the trizol solution that was used in this protocol, is TriPure Isolation Reagent *(Roche)*. This protocol was also tested in two different ways, by removing different amounts of supernatant.

2.2.2.1 The protocol with removing 400 µl of the supernatant

2.2.2.1.a. Materials

- Fresh or frozen tissue
- TissueLyzer
- TissueLyzer adapter set 2x24
- 2 ml microcentrifuge tubes
- Stainless steel beads (3.2 mm)
- Microcentrifuge
- BioDrop
- Pipettes (1000 µl, 200 µl, 2-20 µl)

2.2.2.1.b. Chemicals

- Trizol (TriPure Isolation Reagent)
- Chloroform
- Isopropanol
- 75% ethanol
- RNAse-free water

2.2.2.1.c. Preparations

- 75% ethanol must be prepared by diluting ethanol (96-100%) with distilled water.

2.2.2.1.d. Notes to the protocol

Step 1: Trizol denatures proteins. It creates three layers in the sample: RNA, protein and DNA.

Step 4: After adding chloroform, the 3 layers actually became visible.

Step 5: For centrifugation, the Microlite RF was used.

Step 8: Washing was mainly done with 75% ethanol, because ethanol evaporates a lot better than isopropanol, and it must be absolutely certain that all of the fluid was evaporated before the RNase-free water was added. For each ml of Trizol used in the initial step, 1 ml of ethanol must be used for washing. Because 750 µl of Trizol was used in the initial step, at least 750 µl of ethanol must be used for washing.

Step 11: The RNA was again measured with the BioDrop, and 1.5 µl of each sample was used.

2.2.2.2 The protocol with removing 200 µl of the supernatant

2.2.2.2.a. Materials

The same materials were used as in 2.1.a.

2.2.2.2.b. Chemicals

The same chemicals were used as in 2.1.b.

2.2.2.2.c. Preparations

The same preparations were done as in 2.1.c.

2.2.2.2.d. Notes to the protocol

Step 6: Instead of removing 400 µl of the supernatant, only 200 µl of the supernatant was removed.

Furthermore, the same notes are valid as in 2.1.d.

2.2.3. Mixed protocol *(Appendix 3)*

The last protocol that was tested is a combination of the protocol in 2.2.1, RNA isolation with the RNeasy Plus Micro kit (50) and 2.2.2., RNA isolation with Trizol.

2.2.3.a. Materials

- Fresh or frozen tissue
- TissueLyzer
- TissueLyzer adapter set 2x24
- 2 ml microcentrifuge tubes
- Stainless steel beads (3.2 mm)
- Microcentrifuge
- BioDrop
- Pipettes (1000 µl, 200 µl, 2-20 µl)
- RNeasy minelute spin columns
- 2 ml collection tubes
- 1.5 ml collection tubes

2.2.3.b. Chemicals

- Trizol (TriPure Isolation Reagent)
- Chloroform
- Ethanol
- Buffer RW1
- Buffer RPE
- RNAse-free water

2.2.3.c. Preparations

- Before Buffer RPE can be used, 4 volumes of ethanol (96-100%) must be added. Also, 80% ethanol must be prepared by mixing 24 ml ethanol (96-100%) and 6 ml RNase-free water. 70% ethanol was prepared by diluting ethanol (96-100%) with distilled water.

2.2.3.d. Notes to the protocol

No notes.

2.3. Preparation of cDNA *(Appendix 4)*

Before the actual preparation of cDNA, it is necessary to first do a DNase-reaction, to be absolutely sure that there is no more DNA present in the samples.

2.3.1. The DNase-reaction

2.3.1.a. Materials

- PCR-tubes
- Pipettes (10 µl, 2-20 µl, 200 µl, 1000 µl)

2.3.1.b. Chemicals

- RNA 500 ng
- DNase I buffer 1 µl
- DNase 0,5 µl
- mQ (water)

The total amount of each sample must be 15 µl. Because the amount of RNA in one sample was measured after the isolation, it was known how much RNA was present per milliliter. It could now be calculated how many microliter needed to be add to get to 500 ng of RNA. When this was calculated, it could also be calculated how many microliter of mQ needed to be add to get to the total concentration of 15 µl.

2.3.1.c. Notes to the protocol

- The amounts in 1.b are the amounts for one sample. With the amounts of RNA measured after isolation, more samples could be made for preparing cDNA. In this case, 20 samples were made, so all of the amounts in 1.b must be multiplied by 20 to get to the total amount of chemicals you need.
- When the chemicals are pipetted into the tubes, it is very important to do this in a strict order: mQ → DNase I buffer → RNA → DNase.

2.3.2. Preparing the cDNA *(Appendix 4)*

2.3.2.a. Materials

- PCR-tubes
- Pipettes (10 µl, 2-20 µl, 200 µl)

2.3.2.b. Chemicals

- 5x Buffer Tscript advanced reaction mix 4 µl
- Enzym I script RT 1 µl
- RNA sample from the DNase-reaction 15 µl

2.3.2.c. Notes to the protocol

- In the DNase-reaction, 20 samples were used. 15 µl of each sample was used for the preparation of cDNA.
- The 5x Buffer Tscript advanced reaction mix and the Enzym I script RT were prepared into a mix. Because 20 samples were prepared, the amounts of these chemicals in 2.b must be multiplied by 20. To be sure there was enough of the mix, the amounts were multiplied by 22.
- After preparation of the mix, 5 µl of the mix was add to 15 µl of each RNA sample.

2.4.The qPCR *(Appendix 5)*

The most important step in setting up a protocol for the qPCR is to determine which primers need to be used to get to the optimal result for the respective reference gene/hormone receptor. Primers were tested with different concentrations of cDNA in different amounts of the total sample. Furthermore, different concentrations of RNA were used in the preparation of cDNA.

2.4.1 500 ng RNA in cDNA-reaction; 1 µl cDNA in total concentration of 5 µl

2.4.1.a. Materials

- 384-wels plate
- Pipettes (3 µl, 10 µl, 100 µl, 200 µl)

2.4.1.b. Chemicals

- 5x HOT FIREPol EvaGreen qPCR Mix Plus 1 µl
- Primer F (10 pmol/µl) 0,1 µl
- Primer R (10 pmol/µl) 0,1 µl
- cDNA Template 1 µl
- mQ

Primers tested	Group
RPL4_1F + RPL4_1R ACTB_1F + ACTB_1R PPIA_1F + PPIA_1R GAPDH_1F + GAPDH_1R	**Reference genes**

2.4.1.c. Notes to the protocol

- A total mix was made of 5x HOT FIREPol EvaGreen qPCR Mix Plus and mQ. Before this mix was made, it was decided that the total amount of each eventual sample

must be 5 µl. When decided how much of the DNA would be add to the sample, in this case 1 µl, it can be calculated how much mQ must be add per sample to get to this concentration of 5 µl.

- Because multiple different primers were tested each time, the primers could not be included into the mix. The primers and the cDNA were added after preparation of the mix.
- After the samples were pipetted into the 384-wels plate, the plate was sealed with Biorad microseal® 'B' seal. After sealing, the plate was microcentrifuged very shortly, before it was put in the Biorad CFX384™ Real-Time System C1000 Touch Thermal Cycler for the actual qPCR reaction.
- The following q-PCR program was used:

Cycle step	Temp.	Time	Cycles
Initial activation*	**95°C**	**15 min**	1
Denaturation	95°C	15 s	40
Annealing	60°-65°C	20 s	
Elongation	72°C	20 s	

- Each primer was double tested, once with water and once with cDNA.

2.4.2. 500 ng RNA in cDNA-reaction; 2 µl cDNA in total concentration of 10 µl

2.4.2.a. Materials

The same materials were used as in 2.4.1.a.

2.4.2.b. Chemicals

- The concentration of each sample was raised to 10 µl instead of 5 µl. This means that all of the concentrations in 2.4.1.b. must be multiplied by 2.

Primers tested	Group
Erb_3F + Erb_3R	**Estrogen-receptor**
AR_1F + AR_1R	**Androgen-receptor**
PR_1F + PR_1R PR_3F + PR_3R	**Progesterone-receptor**
GR_1F + GR_1R	**Glucocorticoid-receptor**

2.4.2.c. Notes to the protocol

- The total amount of each sample must be 10 µl instead of 5 µl.

2.4.3. 500 ng RNA in cDNA-reaction; 2 µl cDNA in total concentration of 10 µl

2.4.3.a. Materials

The same materials were used as in 2.4.1.a.

2.4.3.b. Chemicals

- The concentration of each sample was raised to 10 µl instead of 5 µl. This means that all of the concentrations in 2.4.1.b. must be multiplied by 2.

Primers tested	Group
HPRT_1F + HPRT_1R PPIA_1F + PPIA_1R RPL4_1F + RPL4_1R ACTB_1F + ACTB_1R PGK1_1F + PGK1_1R RPS7_1F + RPS7_1R TFRC _1F + TFRC_1R YWHAZ_1F + YWHAZ_1R ACTB_1F2 + ACTB_1R GAPDH_1F + GAPDH_1R	**Reference genes**
Era_1F + Era_1R Erb_3F + Erb_3R	**Estrogen-receptor**
AR_1F + AR_1R	**Androgen-receptor**
PR_1F + PR_1R PR_3F + PR_3R	**Progesterone-receptor**
MR_1F + MR_1R GR_1F + GR_1R	**Corticosterone-receptor**
VT1R_1F + VT1R_1R VT2R_1F + VT2R_1R	**Vasopressin-receptor**

2.4.3.c. Notes to the protocol

- A temperature-gradient was used during the q-PCR. This means that there was no constant annealing temperature of 60-65°C, but this temperature varied between 50-60°C. In this way, some primers were tested at different temperatures, to see if there was an optimum temperature for these primers. The primers were tested at 60°C, 58,4°C, 56,6°C and 50°C.

2.4.4. 500 ng RNA in cDNA-reaction; 7,6 µl cDNA in total concentration of 10 µl

2.4.4.a. Materials

The same materials were used as in 2.4.1.a.

2.4.4.b. Chemicals

- No mQ was used. The concentration of mQ that would normally be added, was replaced by cDNA, so in total 7.6 µl of cDNA was added.
- The concentration of each sample was raised to 10 µl instead of 5 µl. This means that all of the concentration in 2.4.1.b. must be multiplied by 2.

Primers tested	Group
GAPDH_1F + GAPDH_1R	**Reference genes**
Era_3F + Era_3R	**Estrogen-receptor**
MR_1F + MR_1R GR_1F + GR_1R	**Corticosterone-receptor**

2.4.4.c. Notes to the protocol

- A temperature-gradient was again used during the q-PCR. This time the primers were tested at 60°C, 58,4°C, 55,6°C and 52,5°C.

2.4.5. 1000 ng RNA in cDNA-reaction; 2 µl cDNA in total concentration of 10 µl

2.4.5.a. Materials

The same materials were used as in 2.4.1.a.

2.4.5.b. Chemicals

- The concentration of each sample was raised to 10 µl instead of 5 µl. This means that all of the concentrations in 2.4.1.b. must be multiplied by 2.

Primers tested	Group
RPL4_1F + RPL4_1R ACTB_1F + ACTB_1R RPS7_1F + RPS7_1R YWHAZ_1F + YWHAZ_1R	**Reference genes**
MR_1F + MR_1R GR_1F + GR_1R	**Corticosterone-receptor**
VT1R_1F + VT1R_1R	**Vasopressin-receptor**

2.4.5.c. Notes to the protocol

- A temperature-gradient was again used during the q-PCR. This time the primers were tested at 60°C, 58,4°C, 55,6°C and 53,4°C.

3. Results

3.1.RNA isolation

3.1.1. RNeasy Plus Microkit

1.1 Without adding extra carrier RNA

Tube number	RNA (µg/ml)	A260/A280
1	8,068	2,469
2	13,91	1,853
3	17,51	1,576
4	1,411	2,309

1.2 With adding extra carrier RNA

Tube number	RNA (µg/ml)	A260/A280
9	9,253	1,762
10	40,00	1,724
11	11,07	1,765
12	18,66	1,750

3.1.2. Trizol

2.1 With removing 400 µl of the supernatant

Tube number	RNA (µg/ml)	A260/A280
5	109,2	1,475
6	290,0	1,235
7	133,3	1,393
8	120,8	1,398

2.2 With removing 200 µl of the supernatant

Tube number	RNA (µg/ml)	A260/A280
13	2560,00	1,722
14	3192,9	1,695
15	3194,3	1,745
16	3196,1	1,686

3.1.3. Mixed protocol

Tube number	RNA (µg/ml)	A260/A280
17	32,26	1,330
18	89,06	1,275
19	16,27	1,173
20	29,71	1,320

3.2. qPCR

For amplification curves and melt curves, see appendix 6.

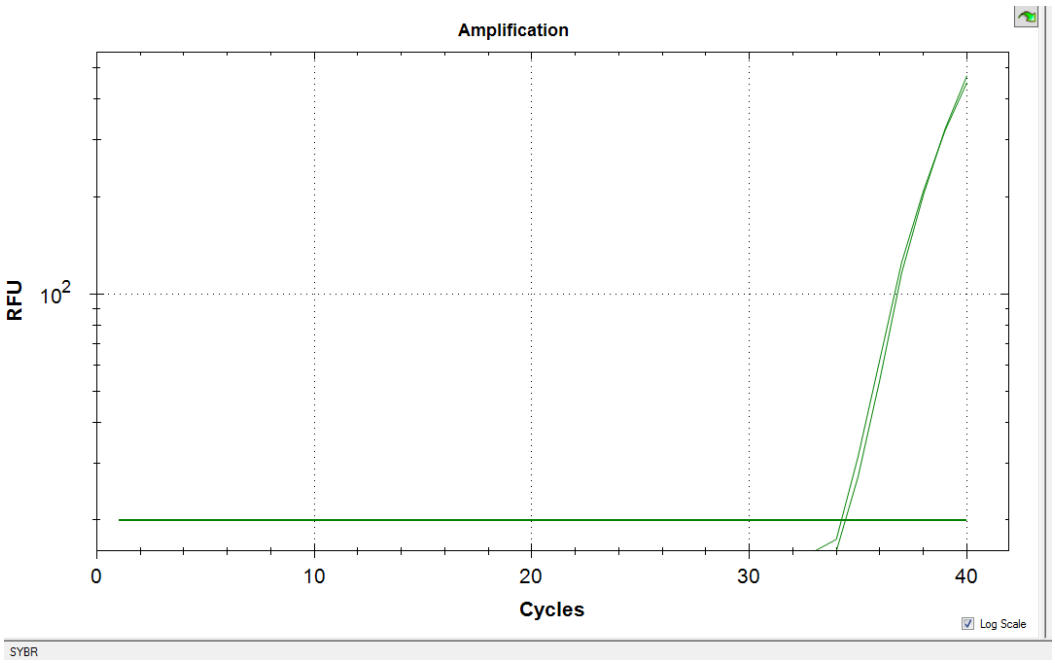
3.2.1. 500 ng RNA in cDNA-reaction; 1 µl cDNA in total concentration of 5 µl

Of the primers that are tested in 2.4.1, the primers that gave an amplification of the cDNA are listed in table 1.

Table 1: ***Working primers for 2.4.1.***

Working primers	Working temperature
RPL4_1F + RPL4_1R PP1A_F + PP1A_R	60°C

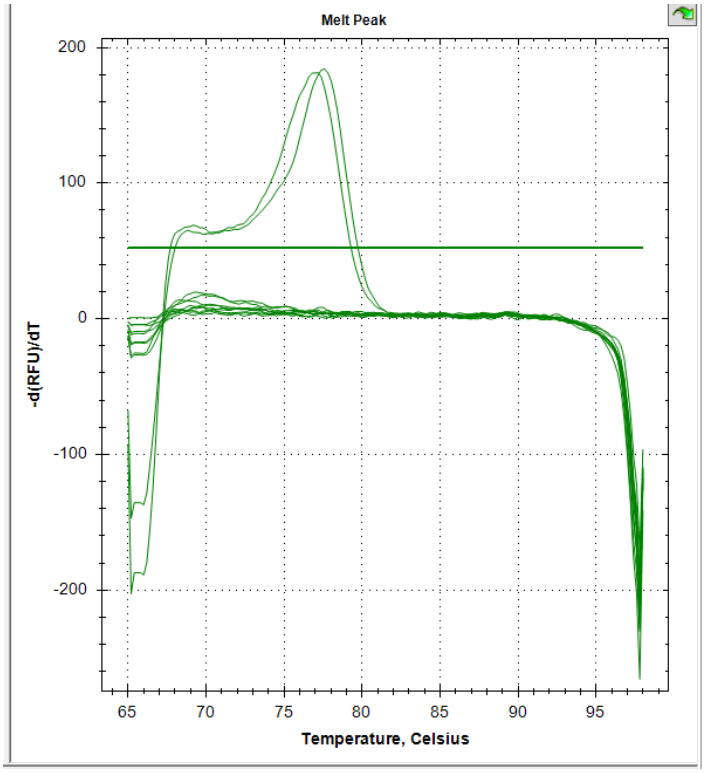
Graphic 1 shows the log scale of the amplification curve of these primers. The horizontal lines in this graphic represent the primers that did not give an amplification of the cDNA. The two lines that begin to raise at approximately 33 and 34 cycles represent the RPL4_1F + RPL4_1R (left line) and PP1A_1F + PP1A_1R (right line). This means that during this cycles the first cDNA was amplified. The purpose of a log scale is that, at a certain point, a maximum amount of amplification is reached. The raising line then becomes a horizontal line. This would be the optimal result for a q-PCR. This graphic shows, that for these primers, this point was not reached.



Graphic 1: ***Log scale of the amplification curve for the primers tested in 2.4.1.***

Graphic 2 shows the melt peak for the primers in 2.4.1. What is important for the melt peak, is that it only shows one peak in each line. More than one peak suggests that the

amplification of the cDNA did not give one single, specific product. Graphic 2 shows that both working primers only gave one peak. This means that these primers produced one single, specific product.



Graphic 2: ***Melt peak for the primers tested in 2.4.1.***

3.2.2. 500 ng RNA in cDNA-reaction; 2 µl cDNA in total concentration of 10 µl

Of the primers that were tested in 2.4.2., the primers that gave an amplification of the cDNA are listed in table 2.

Table 2: ***Working primers for 2.4.2.***

Working primers	Working temperature
GR1_F + GR1_R (water control)	55°C

As shown in table 2, only one water control gave a result. This means that the primers tested in 2.4.2 were not able to amplify the cDNA at the used temperatures. Because the primers did not give a result in amplifying the cDNA, the log scale of the amplification curve and the melt curve for these primers are listed in appendix 6.

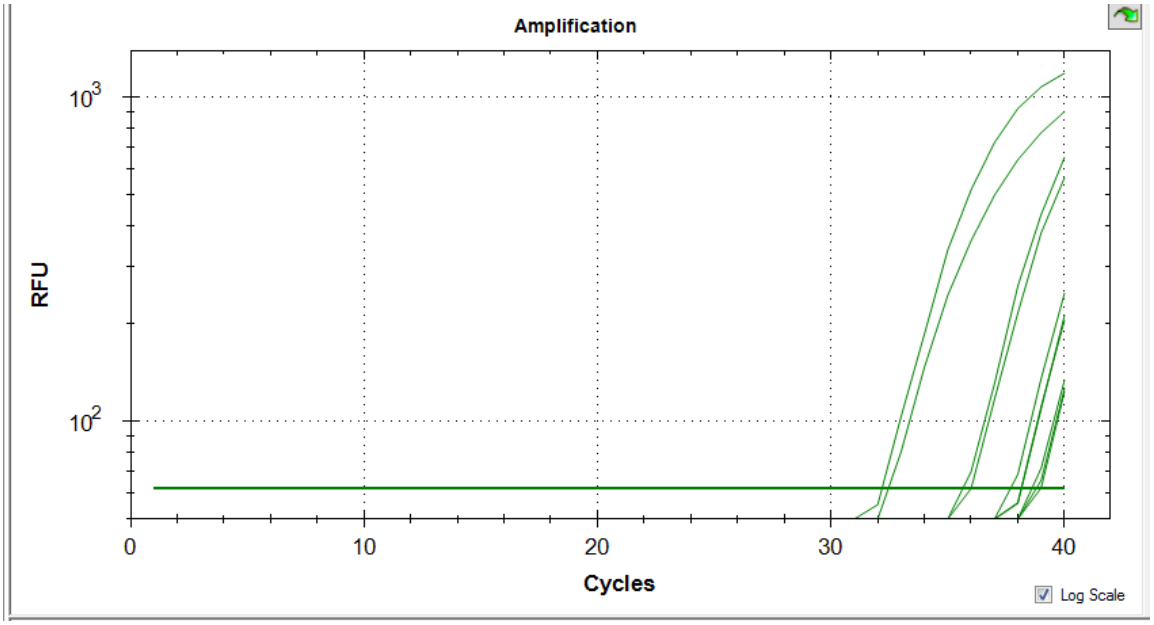
3.2.3. 500 ng RNA in cDNA-reaction; 2 µl cDNA in total concentration of 10 µl

Of the primers that were tested in 2.4.3., the primers that gave an amplification of the cDNA are listed in table 3.

Table 3: ***Working primers for 2.4.3.***

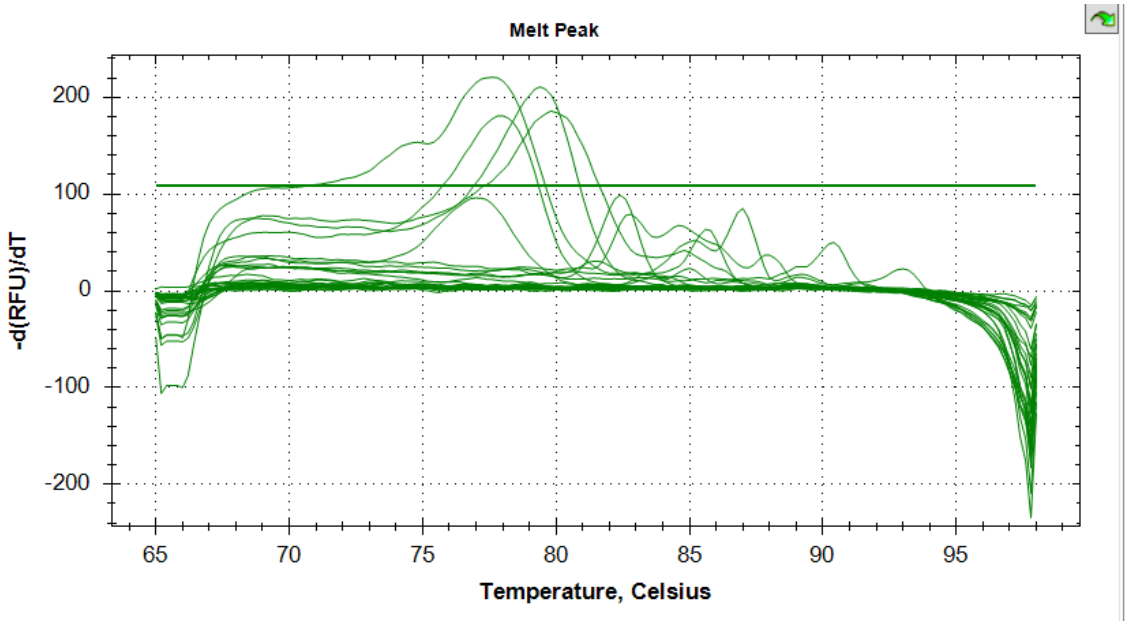
Working primers	Working temperature
RPL4_1F + RPL4_1R VT1R_1F + VT1R_1R	60°C
RPS7_F + RPS7_R YWHAZ_1F + YWHAZ_1R ACTB_1F + ACTB_1R TFRC_1F + TFRC_1R VT2R_1F + VT2R_1R	58,4°C
PR1_F + PR1_R	56,6°C
Erb3_F + Erb3_R Erb3_F + Erb3_R (water control)	50°C

Graphic 3 shows the log scale of the amplification curve for the primers tested in 2.4.3. From the left to the right, the lines represent the following primers: RPL4_1F + RPL4_1R, RPS7_1F + RPS7_1R, YWHAZ_1F + YWHAZ_1R, VT1R_1F + VT1R_1R, PR_1F + PR_1R, ACTB_1F + ACTB_1R, TFRC_1F + TFRC_1R, Erb_3F + Erb_3R, Erb_3F + Erb_3R (water control), VT2R_1F + VT2R_1R.
This graphic shows that the primers started amplifying cDNA in the latest cycles of the qPCR, and no horizontal plateaus occur during this cycles.



Graphic 3: ***Log scale of the amplification curve for the primers tested in 2.4.3.***

Graphic 4 shows the melt peak for the primers tested in 2.4.3. This graphic shows that not all of the primers produced a single, specific product, because some lines clearly show more than one peak.



Graphic 4: ***Melt peak for the primers tested in 2.4.3.***

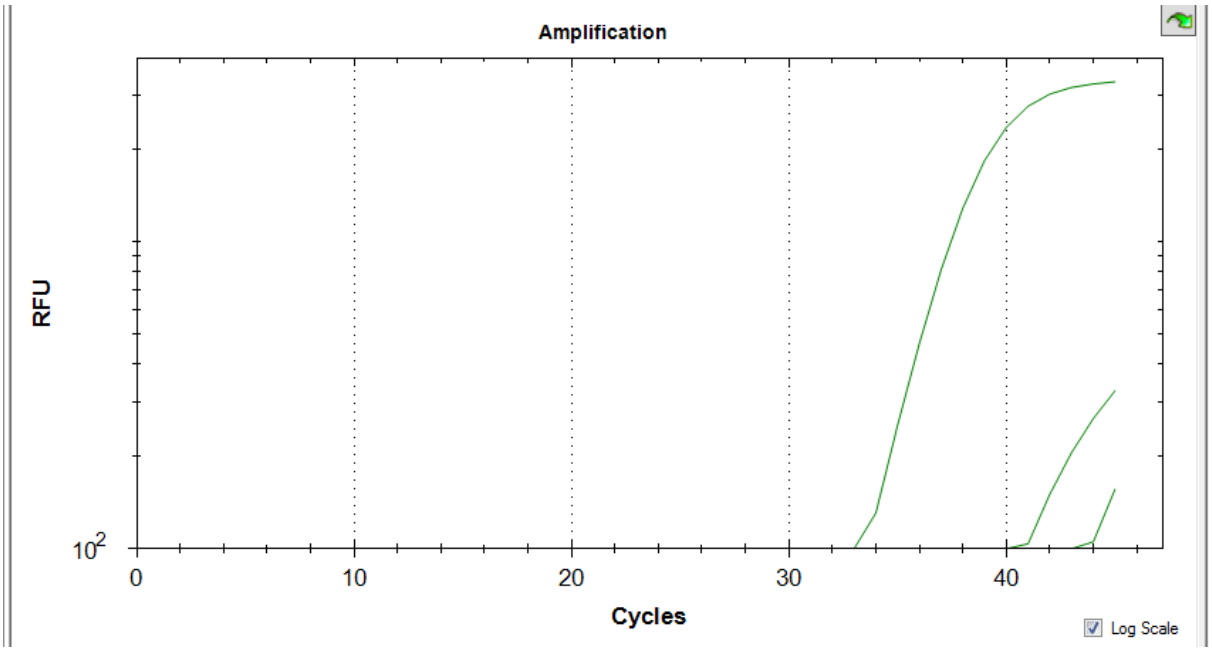
3.2.4. 500 ng of RNA in cDNA-reaction; 7,6 µl cDNA in total concentration of 10 µl

Of the primers that were tested in 2.4.4., the primers that gave an amplification of the cDNA are listed in table 4. One of these results is a result for a water control.

Table 4: ***Working primers in 2.4.4.***

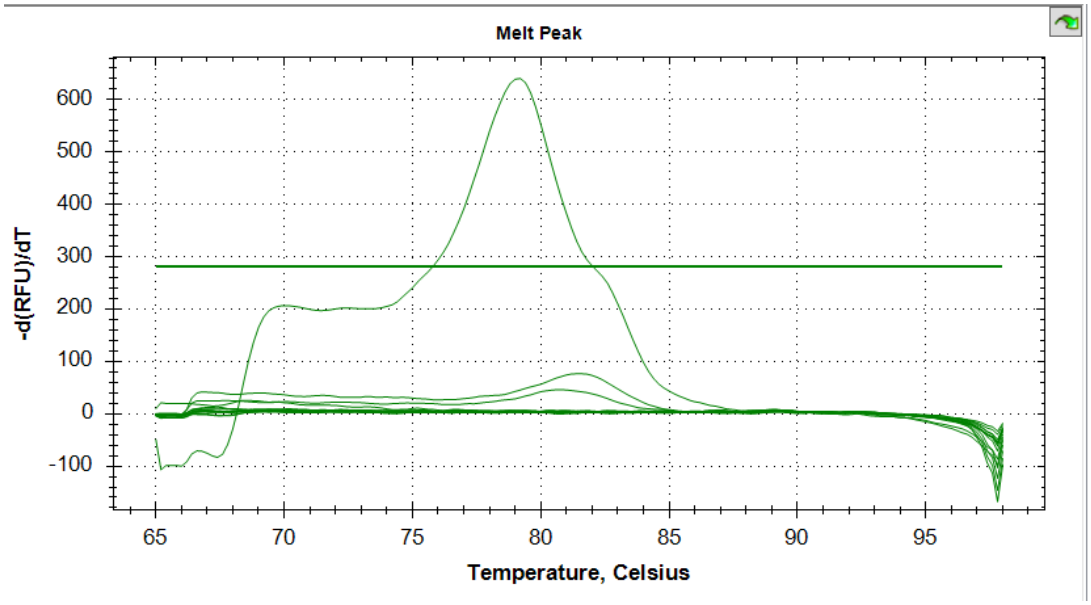
Working primers	Working temperature
Era_3F + Era_3R (water control) Era_3F + Era_3R	58,4°C
Era_3F + Era_3R	55,6°C

Graphic 5 shows the log scale of the amplification curve for the primers tested in 2.4.4. The water control, in this case, gave the first line in this graphic. This line would actually represent a good result, because at the end of the qPCR, the line slightly starts to become horizontal. However, it is not possible that a primers produces DNA when no cDNA is present in the starting sample.



Graphic 5: ***Log scale of the amplification curve for the primers tested in 2.4.4.***

Graphic 6 shows the melt peak for the primers tested in 2.4.4. The large peak shows that one of the primers produced a single, specific product. However, this peak represents the water control. The other two working primers barely give a peak.



Graphic 6: ***Melt peak for the primers tested in 2.4.4.***

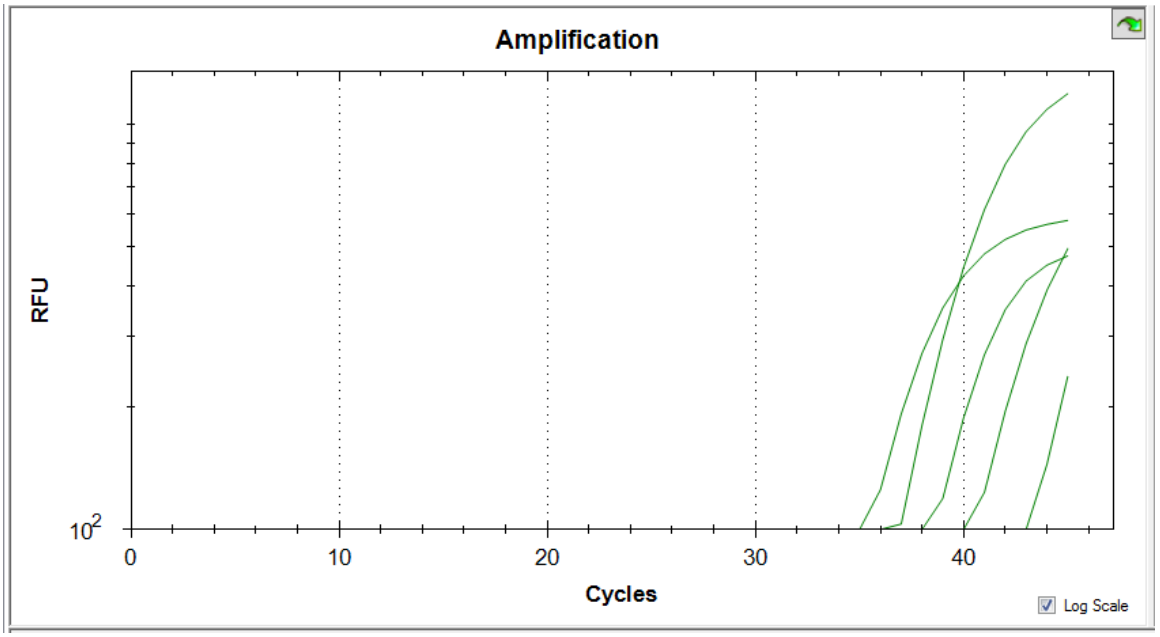
3.2.5. 1000 ng RNA in cDNA-reaction; 2 µl cDNA in total concentration of 10 µl

For the primers that were tested in 2.4.5., the primers that gave an amplification of the cDNA are listed in table 5.

Table 5: ***Working primers for 2.4.5.***

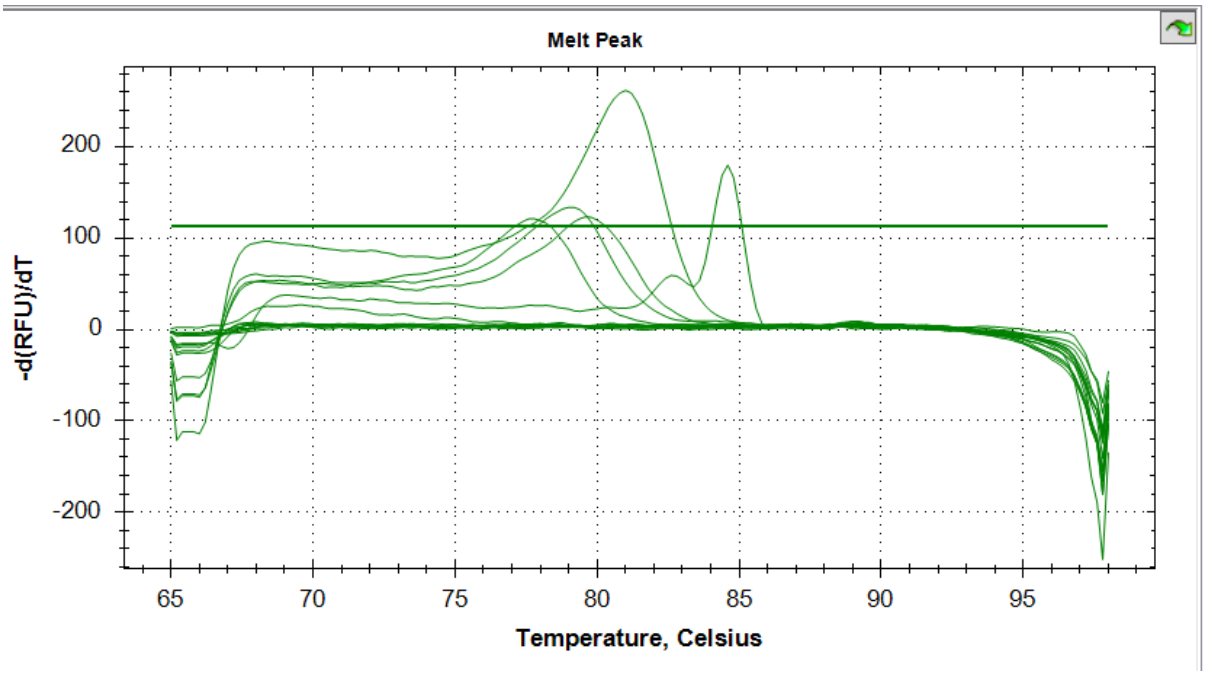
Working primers	Working temperature
RPS7_1F + RPS7_1R RPS7_1F + RPS7_1R (water control) YWHAZ_1F + YWHAZ_1R	58,4°C
RPL4_1F + RPL4_1R ACTB_1F + ACTB_1R	60°C

Graphic 7 shows the log scale of the amplification curve for the primers tested in 2.4.5. From the left to the right, the lines in this graphic represent the following primers: RPS7_1F + RPS7_1R, YWHAZ_1F + YWHAZ_1R, RPL4_1F + RPL4_1R, ACTB_1F + ACTB_1R, YWHAZ_1F + YWHAZ_1R (water control).
Again, all of the primers started amplifying cDNA in late cycles of the q-PCR, and none of the primers gave a clear horizontal plateau.



Graphic 7: ***Log scale of the amplification curve for the primers tested in 2.4.5.***

Graphic 8 shows the melt peak for the primers tested in 2.4.5. This melt peak shows that all of the primers show only one peak, so all of the primers produced one single, specific product, but some of the peaks are very low.



Graphic 8: ***Melt peak for the primers tested in 2.4.5.***

4. Discussion

After testing RNA isolation strictly following the protocols, we made some changes to the protocols. Because the amounts of RNA were very low after isolation with the RNeasy Plus Microkit, we decided to add some extra carrier RNA (included in the kit) to the sample, so that hopefully the amounts of RNA would increase. In some cases this was the case, but not enough to continue to the next steps.

RNA isolation with trizol gave higher concentrations of RNA, but the A260/A280-values of these isolations were very low. To hopefully isolate more RNA and get better A260/A280-values, we decided to remove less of the supernatant. The amounts of RNA indeed increased, and the A260/A280-values became better. Although this was a positive result, the increase was very large, therefore we questioned if this could be correct. A possible explanation for the extreme increase might be that the peak for measuring trizol almost overlaps the peak for measuring RNA, so it could be that there was more trizol left in the eventual sample, because less of the supernatant was removed, and the BioDrop measured trizol as well as RNA. The only fact that did not support this, is that the A260/A280-value was better. When the samples were more contaminated with trizol, you would expect the A260/A280-value to be worse.

We choose to make a mix between the two protocols, because brain tissue contains a lot of fat, and the column membranes from the RNeasy Plus Microkit don't absorb this fat very well. This means that fat contaminates the column membranes, and less RNA can get through. Trizol removes a lot of this fat, so that RNA can better get through the membranes, and higher amounts of RNA are isolated.

The first time primers were tested, 1 µl of cDNA was used in a total concentration of 5 µl. Because the results for amplification were disappointing, we decided to use bigger samples, 10 µl instead of 5 µl. Because the samples became bigger, also more cDNA needed to be add to the samples, 2 µl instead of 1 µl. After these changes, results for amplification still were disappointing. In the total mix we prepared for the q-PCR, not only cDNA was added, but also water. Because the results remained disappointing, we decided to replace the water by cDNA, so more cDNA would be in the samples, 7,6 µl instead of 2 µl.

With preparing the cDNA, at first, 500 ng of RNA was used. We still wanted to try to start amplification sooner, so we figured it might help to use more RNA for preparing the cDNA. That's why the last time primers were tested, we used 1000 ng of RNA for preparing cDNA. After all the little changes we tried, amplification remained disappointing. It still might help to use more RNA in preparing cDNA, but the amount of RNA then needs to be multiplied by a bigger factor than 2. On the other hand, because increasing the used amount of RNA did not give an improvement of amplification at all, it does not seem logical that this will be the case when the amount of RNA would be multiplied by for example a factor of 4.

Something that might be tried in the continuing of this investigation, is to raise the concentration of the primers. When the concentration of the primers is raised, the concentration of cDNA should not be raised, so that the amount of primer becomes bigger compared to the amount of cDNA.

A few times during testing of the primers, water controls gave a result for amplification. The only reasonable explanation seems to be that the water control samples were contaminated with cDNA, for example by using the same pippets. However, this is not possible, because the water control samples and the samples with cDNA were pipetted in different rooms, with different pipettes.
During testing of the primers, it became clear that the primers for reference genes gave better results than the primers for the steroid receptors. One explanation could be that the primers for reference genes are better able to bind to the cDNA and the primers for the steroid receptors do not yet have the optimal nucleotide sequence to bind to the cDNA. After all, no primers are known for steroid receptors in quail, the used primers are only found in chicken. Another explanation might be that the steroid receptors are simply present in such low concentrations, that the primers have low concentrations of cDNA to bind to.

5. Conclusion

As shown in this study, company protocols are not always most suitable for the used tissues. The protocol for RNA isolation with the RNeasy Microkit Plus (50) gave very low outcomes for RNA quantity, and was therefore disregarded.
RNA isolation with Trizol on the other hand, gave very high outcomes for RNA quantity. However, both of these outcomes did not seem very reliable. When a mixed protocol was designed, the outcomes for RNA quantity seemed more logical, and thus more reliable. I therefore suggest to continue with the use of the combined protocol for further testing and finalizing RNA extraction and qPCR of Japanese quail brain samples.

For testing the primers, no optimal results are yet acquired. Because the results for amplification were so disappointing, it is not possible to conclude if the used primers have the right nucleotide sequences. There need to be searched for a way to start amplification of cDNA in earlier cycles of the qPCR, so it can be better judged if the right primers are used. I would suggest to first try if better amplification results can be reached by increasing the used amount of the primers, without changing the used amount of cDNA.

Appendix

Appendix 1

Protocol: Purification of Total RNA from Animal and Human Tissues

This protocol is for the purification of total RNA from easy-to-lyse animal and human tissues. For total RNA purification from frozen, microdissected tissue samples, see page 31.

Determining the correct amount of starting material

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. A maximum amount of 5 mg fresh or frozen tissue or 2–3 mg RNA*later* or Allprotect stabilized tissue (which is partially dehydrated) can generally be processed. For most tissues, the DNA removal capacity of the gDNA Eliminator spin column, the RNA binding capacity of the RNeasy MinElute spin column, and the lysing capacity of Buffer RLT Plus will not be exceeded by these amounts. Typical RNA yields from various tissues are given in Table 2 (page 12).

For maximum RNA yields from liver, 50% ethanol (instead of 70% ethanol) should be used in step 4 of the procedure.

Some tissues such as spleen, parts of brain, lung, and thymus tend to form precipitates during the procedure. However, this does not affect RNA purification.

Do not overload the gDNA Eliminator spin column, as this will lead to copurification of DNA with RNA. Do not overload the RNeasy MinElute spin column, as this will significantly reduce RNA yield and quality.

Weighing tissue is the most accurate way to quantitate the amount of starting material. As a guide, a 1.5 mm cube (3.4 mm^3) of most animal tissues weighs 3.5–4.5 mg.

Important points before starting

- If using the RNeasy Plus Micro Kit for the first time, read "Important Notes" (page 11).
- If preparing RNA for the first time, read Appendix A (page 39).
- If using the TissueRuptor, ensure that you are familiar with operating it by referring to the *TissueRuptor User Manual* and *TissueRuptor Handbook*.
- If using the TissueLyser, ensure that you are familiar with operating it by referring to the operating instructions and *TissueLyser Handbook*.
- For optimal results, stabilize harvested tissues immediately in RNA*later* RNA Stabilization Reagent (see the *RNAlater Handbook*) or Allprotect Tissue Reagent (see the *Allprotect Tissue Reagent Handbook*). Tissues can be stored in the reagent at 37°C for up to 1 day, at 15–25°C for up to 7 days,

or at 2–8°C for up to 4 weeks (RNA*later*) or 6 months (Allprotect). Alternatively, tissues can be archived at –15 to –30°C or –80°C.

- Fresh, frozen, or RNA*later*/Allprotect stabilized tissue can be used. Tissues can be stored at –70°C for several months. Flash-freeze tissues in liquid nitrogen, and immediately transfer to –70°C. Do not allow tissues to thaw during weighing or handling prior to disruption in Buffer RLT Plus. Homogenized tissue lysates from step 2 can also be stored at –70°C for several months. Incubate frozen lysates at 37°C in a water bath until completely thawed and salts are dissolved before continuing with step 3. Avoid prolonged incubation, which may compromise RNA integrity.
- Buffer RLT Plus and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 6 for safety information.
- Perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

Things to do before starting

- β-Mercaptoethanol (β-ME) must be added to Buffer RLT Plus before use. Add 10 µl β-ME per 1 ml Buffer RLT Plus. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT Plus containing β-ME can be stored at room temperature (15–25°C) for up to 1 month. Alternatively, add 20 µl of 2 M dithiothreitol (DTT) per 1 ml Buffer RLT Plus. The stock solution of 2 M DTT in water should be prepared fresh, or frozen in single-use aliquots. Buffer RLT Plus containing DTT can be stored at room temperature for up to 1 month.
- When processing less than about 2 µg tissue, carrier RNA may be added to the lysate before homogenization (see "Carrier RNA", page 15). Before using for the first time, dissolve the carrier RNA (310 µg) in 1 ml RNase-free water. Store this stock solution at –15 to –30°C, and use it to make fresh dilutions for each set of RNA preps. The concentration of this stock solution is 310 µg/ml (i.e., 310 ng/µl). To make a working solution (4 ng/µl) for 10 preps, add 5 µl stock solution to 34 µl Buffer RLT Plus and mix by pipetting. Add 6 µl of this diluted solution to 54 µl Buffer RLT Plus to give a working solution of 4 ng/µl. Add 5 µl of this solution to the lysate in step 2. **Do not add the carrier RNA to the lysate if purifying RNA for use in oligo-dT–based amplification.**
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

- Before using the kit for the first time, prepare 80% ethanol by mixing 24 ml ethanol (96–100%) and 6 ml RNase-free water (supplied). The procedure also requires 70% ethanol, which can be prepared by diluting ethanol (96–100%) with distilled water (not supplied).
- Buffer RLT Plus may form a precipitate during storage. If necessary, redissolve by warming and then place at room temperature.

Procedure

1. **Excise the tissue sample from the animal or remove it from storage. Determine the amount of tissue. Do not use more than 5 mg. Proceed immediately to step 2.**

   Weighing tissue is the most accurate way to determine the amount. If necessary, cut the tissue on a clean surface and weigh the piece to be used.

   For RNA*later* or Allprotect stabilized tissues: Remove the tissue from the stabilization reagent using forceps and be sure to remove any crystals that may have formed. RNA in RNA*later* or Allprotect stabilized tissues is protected during cutting and weighing of tissues at ambient temperature (15–25°C). It is not necessary to cut the tissues on ice or dry ice or in a refrigerated room. Remaining tissues can be stored in RNA*later* or Allprotect Reagent. Previously stabilized tissues can be stored at –80°C without the reagent.

   For unstabilized fresh or frozen tissues: RNA in harvested tissues is not protected until the tissues are treated with RNA*later* or Allprotect Reagent, flash-frozen, or disrupted and homogenized in step 2. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible. Remaining fresh tissues can be placed into RNA*later* Reagent to stabilize RNA or in Allprotect Tissue Reagent to stabilize DNA, RNA, and protein. However, previously frozen tissues thaw too slowly in the reagent, preventing the reagent from diffusing into the tissues quickly enough to prevent RNA degradation.

2. **Disrupt the tissue and homogenize the lysate in Buffer RLT Plus (do not use more than 5 mg tissue) according to step 2a, 2b, or 2c.**

   See "Disrupting and homogenizing starting material", page 13, for more details on disruption and homogenization.

   Note: Ensure that β-ME (or DTT) is added to Buffer RLT Plus before use (see "Things to do before starting").

   Note: If processing <2 µg tissue, 20 ng carrier RNA (5 µl of a 4 ng/µl solution) may be added to the lysate before homogenization. Prepare the carrier RNA as described in "Things to do before starting".

After storage in RNA*later* or Allprotect Reagent, tissues may become slightly harder than fresh or thawed tissues. Disruption and homogenization using standard methods is usually not a problem.

Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the gDNA Eliminator and RNeasy MinElute spin columns. Homogenization with the TissueRuptor or TissueLyser generally results in higher RNA yields than with other methods.

2a. Disruption and homogenization using the TissueRuptor:

- **Place the tissue in a suitably sized vessel. Add 350 µl Buffer RLT Plus.**

  Note: Use a suitably sized vessel with sufficient extra headspace to accommodate foaming, which may occur during homogenization.

  Generally, round-bottomed tubes allow more efficient disruption and homogenization than conical-bottomed tubes.

- **Place the tip of the disposable probe into the vessel and operate the TissueRuptor at full speed until the lysate is homogeneous (usually 30 s). Proceed to step 3.**

  Note: To avoid damage to the TissueRuptor and disposable probe during operation, make sure the tip of the probe remains submerged in the buffer.

  Foaming may occur during homogenization. If this happens, let the homogenate stand at room temperature for 2–3 min until the foam subsides before continuing with the procedure.

2b. Disruption and homogenization using the TissueLyser:

- **Place the tissues in 2 ml microcentrifuge tubes containing one stainless steel bead (5 mm mean diameter).**

  If handling fresh or frozen tissue samples, keep the tubes on dry ice.

- **Place the tubes at room temperature. Immediately add 350 µl Buffer RLT Plus per tube.**
- **Place the tubes in the TissueLyser Adapter Set 2 x 24.**
- **Operate the TissueLyser for 2 min at 20 Hz.**

  The time depends on the tissue being processed and can be extended until the tissue is completely homogenized.

- **Rearrange the collection tubes so that the outermost tubes are innermost and the innermost tubes are outermost. Operate the TissueLyser for another 2 min at 20 Hz.**

  Rearranging the tubes allows even homogenization.

- **Carefully pipet the lysates into new microcentrifuge tubes (not supplied). Proceed to step 3.**

Do not reuse the stainless steel beads.

2c. Disruption using a mortar and pestle followed by homogenization using a QIAshredder homogenizer or a needle and syringe:

- **Immediately place the weighed tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle.**
- **Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.**
- **Add 350 µl Buffer RLT Plus.**
- **Pipet the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Alternatively, pass the lysate at least 5 times through a blunt 20-gauge needle fitted to an RNase-free syringe. Proceed to step 3.**

3. Centrifuge the lysate for 3 min at full speed. Carefully remove the supernatant by pipetting, and transfer it to a gDNA Eliminator spin column placed in a 2 ml collection tube (supplied). Centrifuge for 30 s at ≥8000 x g (≥10,000 rpm). Discard the column, and save the flow-through.

This step is important, as it removes insoluble material that could clog the gDNA Eliminator spin column and interfere with DNA removal. In some preparations, very small amounts of insoluble material will be present after the 3-min centrifugation, making the pellet invisible.

Note: Make sure that no liquid remains on the column membrane after centrifugation. If necessary, repeat the centrifugation until all liquid has passed through the membrane.

4. Add 1 volume (usually 350 µl) of 70% ethanol to the flow-through from step 3, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 5.

Note: The volume of 70% ethanol to add may be less than 350 µl if some lysate was lost during homogenization and DNA removal.

Note: Precipitates may be visible after addition of ethanol, but this does not affect the procedure.

Note: For maximum RNA yields from liver, use 50% ethanol instead of 70% ethanol.

5. **Transfer the sample, including any precipitate that may have formed, to an RNeasy MinElute spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flowthrough.***

   Optional: If recovery of protein is desired, keep the flow-through on ice and follow steps E1–E5 in Appendix E on page 47.

   Reuse the collection tube in step 6.

6. **Add 700 µl Buffer RW1 to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.***

   Reuse the collection tube in step 7.

   Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

7. **Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.**

   Reuse the collection tube in step 8.

   Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting").

8. **Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the collection tube with the flow-through.**

   Prepare the 80% ethanol with ethanol (96–100%) and the RNase-free water supplied with the kit.

   Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

9. **Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the collection tube with the flow-through.**

   To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

* Flow-through contains Buffer RLT Plus or Buffer RW1 and is therefore not compatible with bleach. See page 6 for safety information.

It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

10. **Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.**

    As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be reduced by approximately 20%. Do not elute with less than 10 µl RNase-free water, as the spin column membrane will not be sufficiently hydrated.

    The dead volume of the RNeasy MinElute spin column is 2 µl: elution with 14 µl RNase-free water results in a 12 µl eluate.

    For RT-PCR and real-time RT-PCR with the purified RNA, QIAGEN offers a range of optimized, ready-to-use kits that provide highly specific and sensitive results. For details, visit www.qiagen.com/PCR . For whole transcriptome amplification (WTA) of limited amounts of RNA, we recommend the QuantiTect Whole Transcriptome Kit. For details, visit www.qiagen.com/goto/WTA .

Appendix 2

RNA isolation with TriPure Isolation Reagent

1. Add 750 µl of the TriPure Isolation Reagent (Trizol) and a stainless steel bead of 3.2 mm mean diameter to the tubes containing your sample.
2. Put the tubes in the TissueLyzer for 5 min on 25 Hz.
3. Rearrange the tubes so that the outermost tubes become innermost and the innermost tubes become outermost. Again, put the tubes in the TissueLyzer for 5 min on 25 Hz.
4. Wait for 5 min. After those 5 min, add 200 µl of chloroform to each tube and shake each tube for 15 sec.
5. Wait for 10 min. After those 10 min, centrifuge for 20 min on maximum speed (13400 rpm) at 4°C.
6. After centrifugation, carefully remove the supernatant (about 400 µl) and put it in a new tube.
7. Add 0.5 ml of isopropanol to each new tube, and wait for 10 min. After those 10 min, centrifuge for 20 min on maximum speed (13400 rpm) at 4°C.
8. After centrifugation, add 75% ethanol to each tube. For each ml of Trizol used in step 1, you need to add at least 1ml of 75% ethanol. Centrifuge for 10 min at maximum speed (13500) at 4°C.
9. After centrifugation, carefully remove the supernatant, and wait for 10 min.
10. Add 10 µl of RNase-free water.
11. Mix the tubes for 10 min.

Appendix 3

Mixed Trizol/Column Protocol

1. Add 750 µl of Trizol and a stainless steel bead of 3.2 mm mean diameter to the tubes containing your sample.
2. Put the tubes in the TissueLyzer for 2 min on 20 Hz.
3. Rearrange the tubes so that the outermost tubes become innermost and the innermost tubes become outermost. Again, put the tubes in the TissueLyzer for 2 min on 20 Hz.
4. Wait for 5 min. After those 5 min, add 200 µl of chloroform to each tube and shake each tube for 15 sec.
5. Wait for 10 min. After those 10 min, centrifuge for 20 min on maximum speed (13400 rpm) at 4°C.
6. After centrifugation, carefully remove the supernatant (about 400 µl) and put it in a new tube.
7. Add 400 µl of 70% ethanol.
8. Now continue with step 5 t/m 10 of the RNeasy Plus Micro Handbook (page 29-30).

Appendix 4

BIO-RAD

iScript™ Advanced cDNA Synthesis Kit for RT-qPCR

Catalog #	Description
1725037	iScript Advanced cDNA Synthesis Kit for RT-qPCR, 25 x 20 µl reactions
1725038	iScript Advanced cDNA Synthesis Kit for RT-qPCR, 100 x 20 µl reactions

For research purposes only.

Introduction

The iScript Advanced cDNA Synthesis Kit for RT-qPCR is an enhanced formulation that offers increased data throughput from a single 20 µl reverse transcription (RT) reaction for real-time quantitative PCR (qPCR). This two-tube kit enables superior capacity as well as a wide linear dynamic range for reverse transcription.

- **Increase qPCR data throughput and cost effectiveness from a single reaction** — cDNA synthesized from higher input RNA allows the analysis of a large number of target genes
- **Reduce interassay variability** — higher yields of cDNA offer flexibility of qPCR replicates
- **Detect low-level target genes** — uncompromised sensitivity even with lower input RNA amounts, in which sample is limited

Storage and Stability

Store at –20°C. Guaranteed for 12 months at –20°C in a constant temperature freezer.

Kit Contents

Reagent	Description
5x iScript Advanced Reaction Mix	5x reaction mix with dNTPs, oligo(dT), and random primers
iScript Advanced Reverse Transcriptase	RNase H+ Moloney murine leukemia virus (MMLV) reverse transcriptase and RNase inhibitor
Nuclease-free water	1.5 ml

Reaction Setups

Reaction Setup for a Single cDNA Synthesis Reaction

For optimal results, reactions should be assembled on ice using appropriate reaction vessels.

Component	Volume per Reaction, µl
5x iScript Advanced Reaction Mix	4
iScript Advanced Reverse Transcriptase	1
RNA template (100 fg–7.5 µg)*	Variable
Nuclease-free water	Variable
Total volume	20

* Input RNA amounts must be optimized based on target gene abundance and sample availability. Ensuring the quality and purity of the RNA sample is essential for achieving the highest capacity.

Reaction Setup for Multiple cDNA Synthesis Reactions

The example below shows a master mix preparation for ten reactions with 5 µl input RNA and enough excess master mix to accommodate loss during pipetting (in this case 12 reactions). For optimal results, reactions should be assembled on ice using appropriate reaction vessels.

Note: If more reactions are required, scale up appropriately. The volumes of components provided in 25- and 100-reaction kits does not take into account the preparation of excess master mix.

Component	Volume per Reaction, µl
5x iScript Advanced Reaction Mix	48
iScript Advanced Reverse Transcriptase	12
Nuclease-free water	120
Total volume	180

1. Prepare the reverse transcription master mix as indicated in the table above. Mix thoroughly by pipetting up and down several times.
2. Add 15 µl of the prepared master mix to 5 µl input total RNA for each reverse transcription reaction.
3. Adjust the volume of water if the input RNA volume differs from the above example. The final volume of master mix to be pipetted into each reaction should also be adjusted.

Reaction Protocol

Incubate the complete reaction mix in a thermal cycler using the following protocol:

Reverse transcription	20 min at 46°C
RT inactivation	1 min at 95°C

Recommendations for qPCR

- For input RNA (1.0 µg–7.5 µg): cDNA generated with this kit must be diluted at least 10-fold in 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, or nuclease-free water prior to use in qPCR
- For input RNA less than 1 µg: cDNA generated with this kit can be used directly in qPCR
- Optimum cDNA dilution must be determined based on target gene abundance and qPCR chemistry
- The volume of cDNA synthesis reaction used must not exceed 10% of the qPCR volume

Recommendations for the Use of No-RT Control

- Contamination by genomic DNA carryover in RNA samples can be tested by setting up a no-RT control reaction
- To set up a no-RT control reaction, simply replace the reverse transcriptase volume with nuclease-free water
- The same amount of total RNA should be used in both the RT and no-RT reactions to ensure similar carryover of cDNA synthesis components into a qPCR reaction

Recommendations for cDNA Archiving

cDNA can be stored at –20°C either undiluted or diluted in 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA.

Related Products

Catalog #	Description
Reverse Transcription Reagents for Real-Time qPCR	
1708840	iScript Reverse Transcription Supermix for RT-qPCR
1708890	iScript cDNA Synthesis Kit
1708896	iScript Select cDNA Synthesis Kit
1725034	iScript gDNA Clear cDNA Synthesis Kit
Reagents for Real-Time qPCR	
1725270	SsoAdvanced™ Universal SYBR® Green Supermix
1725280	SsoAdvanced Universal Probes Supermix
1725120	iTaq™ Universal SYBR® Green Supermix
1725130	iTaq Universal Probes Supermix
1725160	SsoAdvanced PreAmp Supermix

Visit bio-rad.com/web/advancedcDNA for more information.

Appendix 5

5x HOT FIREPol® EvaGreen® qPCR Mix Plus (no ROX)

Cat. No.	Pack Size	Conc. ($MgCl_2$)
08-25-0000S	0.2 ml SAMPLE (50 reactions)	12.5 mM
08-25-00001	1 ml (250 reactions)	12.5 mM
08-25-00008	8 ml (2000 reactions)	12.5 mM
08-25-00020	20 ml (5000 reactions)	12.5 mM

For *in vitro* use only

Description:

HOT FIREPol® EvaGreen® qPCR Mix Plus (no ROX) is an optimised ready-to-use solution for real-time quantitative PCR assays, incorporating EvaGreen® dye. It comprises all the components necessary to perform qPCR: HOT FIREPol® DNA Polymerase, ultrapure dNTPs, $MgCl_2$ and EvaGreen® dye. The user simply needs to add water, template and primers.
HOT FIREPol® DNA Polymerase is activated by a 15 min incubation step at 95°C. This prevents extension of non-specifically annealed primers and primer-dimers formed at low temperatures during qPCR setup.

Applications:

- Detection and quantification of DNA and cDNA targets
- Profiling gene expression
- Microbial detection
- Viral load determination

Mix Composition:

- **HOT FIREPol® DNA Polymerase**
- **5x EvaGreen® qPCR buffer**
- **12.5 mM $MgCl_2$**
  1x PCR solution – 2.5 mM $MgCl_2$
- **dNTPs**
- **EvaGreen® dye**
- **No ROX dye**

EvaGreen® Dye:

EvaGreen® is a DNA-binding dye with many features that make it a superior alternative to SYBR® Green I for qPCR. Apart from having similar spectra, EvaGreen® has three important features that set it apart from SYBR® Green I: EvaGreen® has much less PCR inhibition, is extremely stable dye and has been shown to be nonmutagenic and noncytotoxic. EvaGreen® is compatible with all common real-time PCR cyclers – simply select the standard settings for SYBR® Green or FAM!

Shipping and Storage conditions:

Routine storage: -20°C
Shipping and temporary storage for up to 1 month at room temperature has no detrimental effects on the quality of HOT FIREPol® EvaGreen® qPCR Mix Plus (no ROX).

Recommended qPCR reaction mix:

Component	Volume	Final conc.
5x HOT FIREPol® EvaGreen® qPCR Mix Plus	4 µl	1x
Primer Forward (10 pmol/µl)	0.16-0.5 µl	80-250 nM
Primer Reverse (10 pmol/µl)	0.16-0.5 µl	80-250 nM
DNA template [1]	variable[1]	variable[1]
H_2O PCR grade	up to 20 µl	
Total	**20 µl**	

[1] Conc. of cDNA 0.1 pg/µl -10 ng/µl ; gDNA 10 pg/µl – 4 ng/µl

Recommended qPCR cycles:

Cycle step	Temp.	Time	Cycles
Initial activation*	**95°C**	**15 min**	1
Denaturation	95°C	15 s	40
Annealing	60°-65°C	20 s	
Elongation	72°C	20 s	

* To activate the polymerase, include an incubation step **at 95°C for 15 minutes** at the beginning of the qPCR cycle.

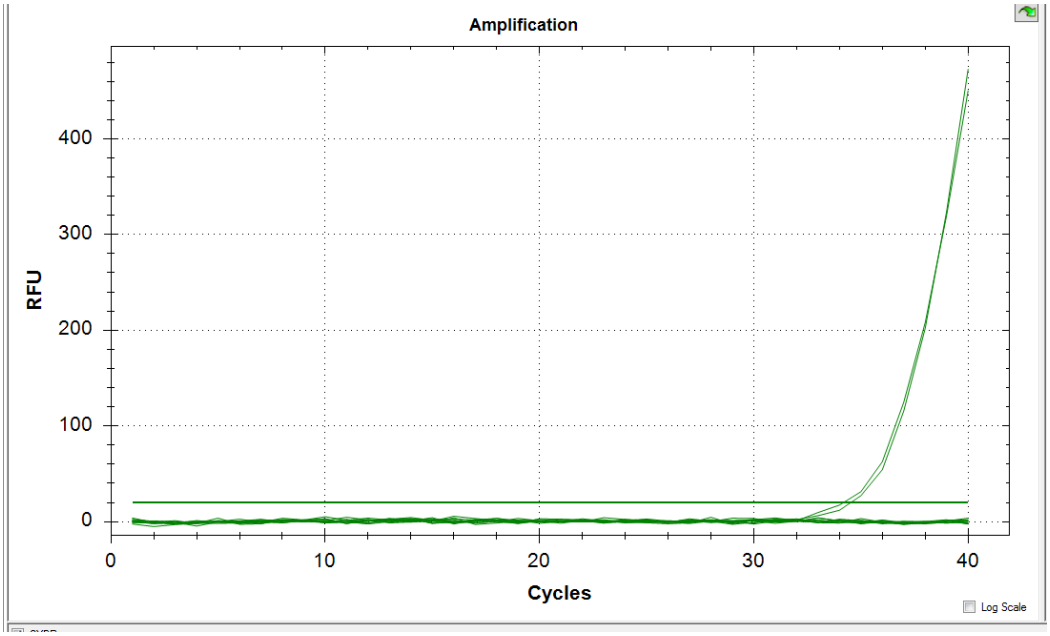
In order to prevent contamination, we recommend you to setup the reaction under laminar or in PCR box.

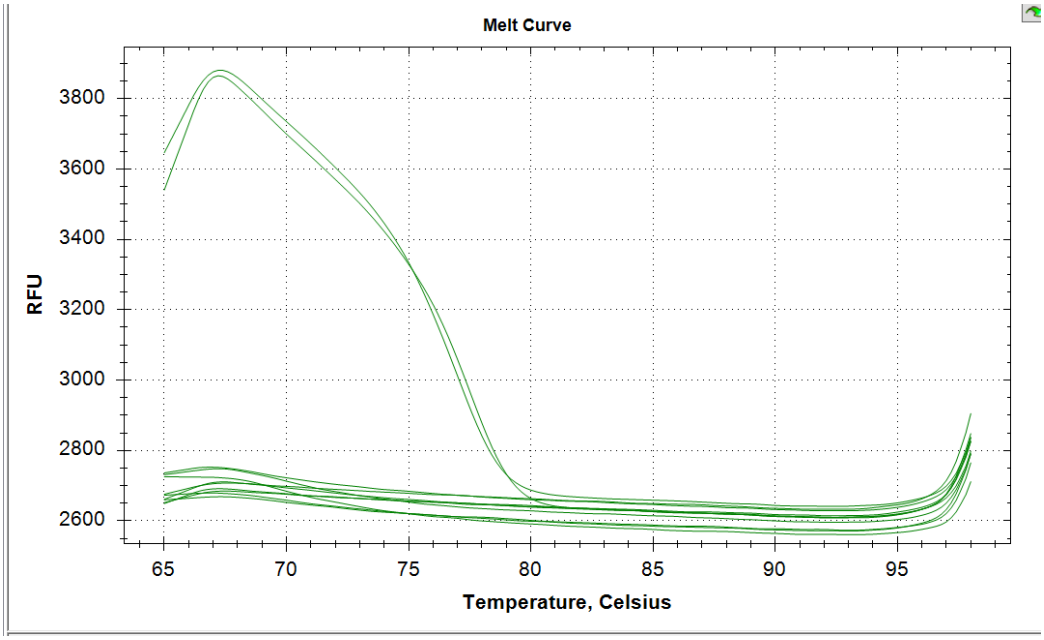
Safety warnings and precautions:

This product and its components should be handled only by persons trained in laboratory techniques. It is advisable to wear suitable protective clothing, such as laboratory overalls, gloves and safety glasses. Care should be taken to avoid contact with skin or eyes. In case of contact with skin or eyes, wash immediately with water.

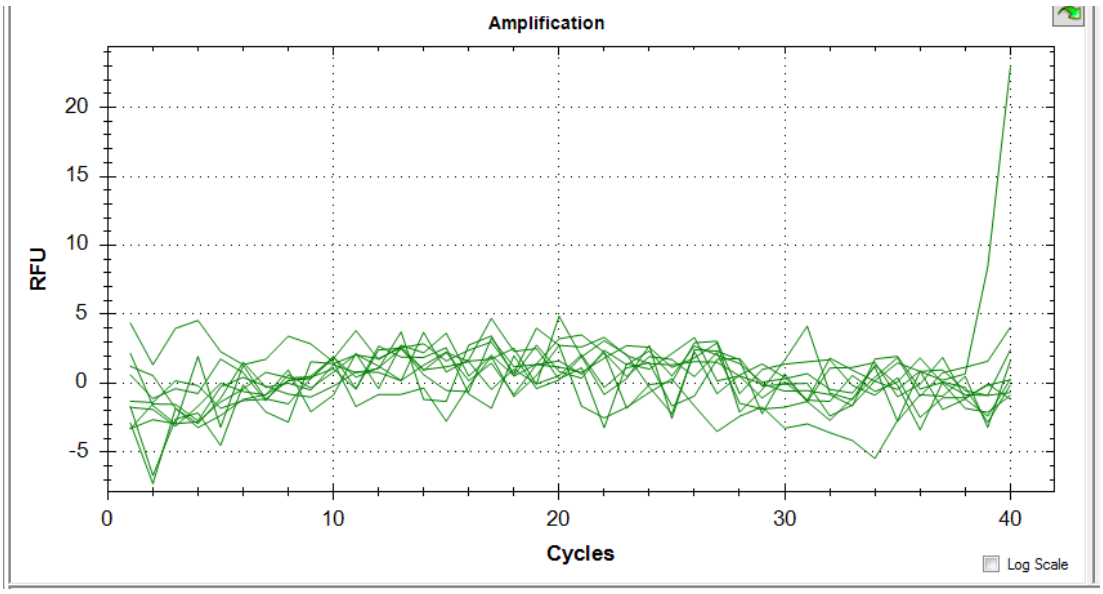
Appendix 6

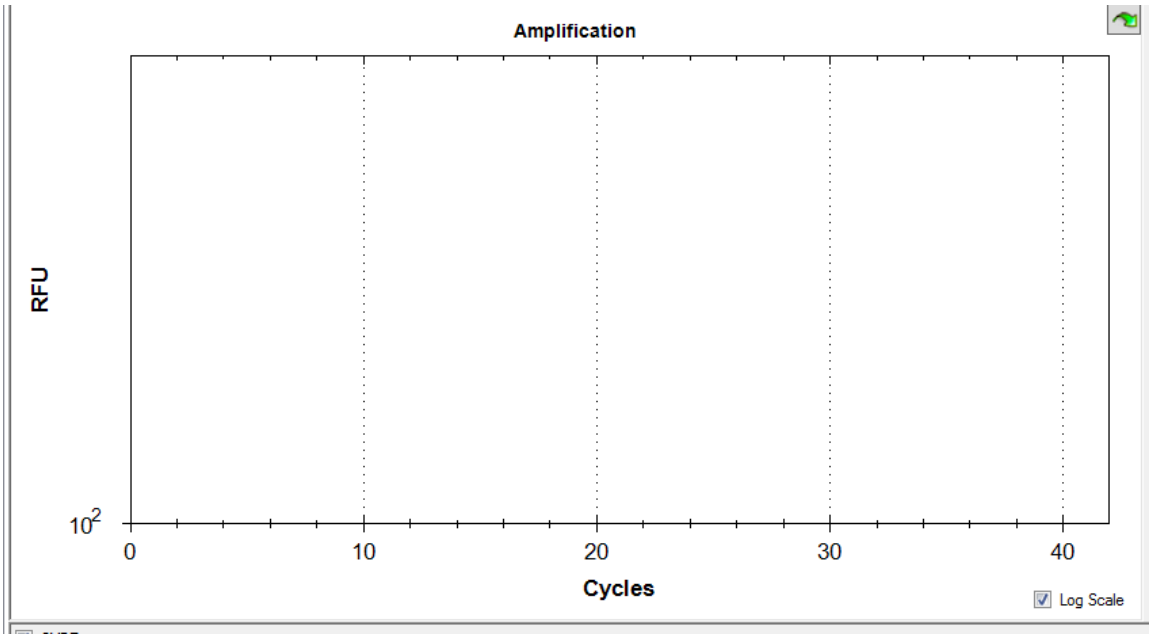
6.1 500 ng RNA in cDNA-reaction; 1 µl cDNA in total concentration of 5 µl

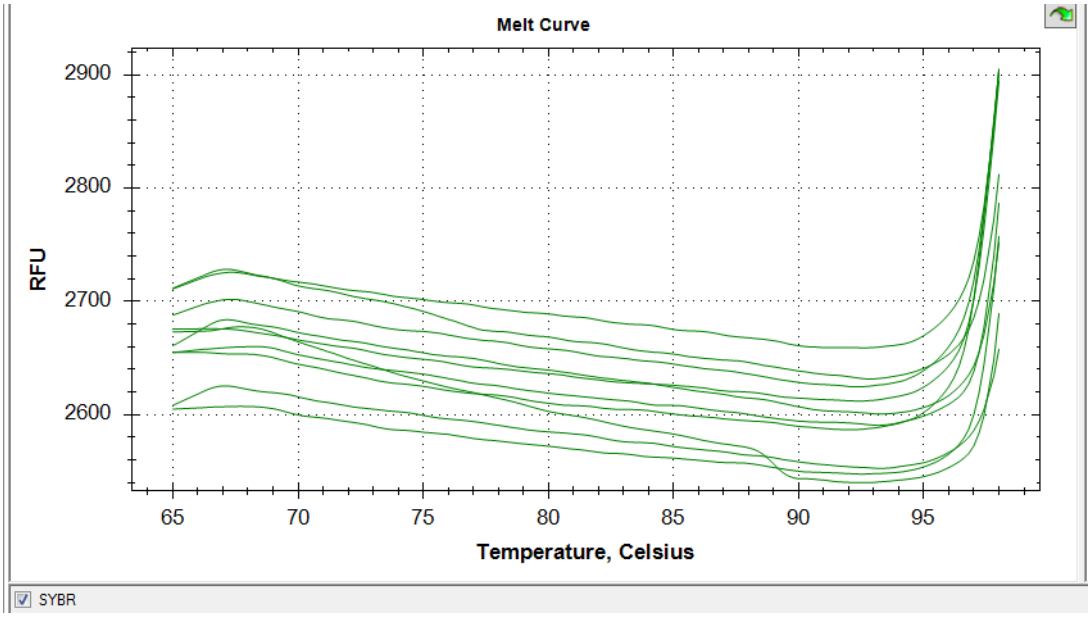


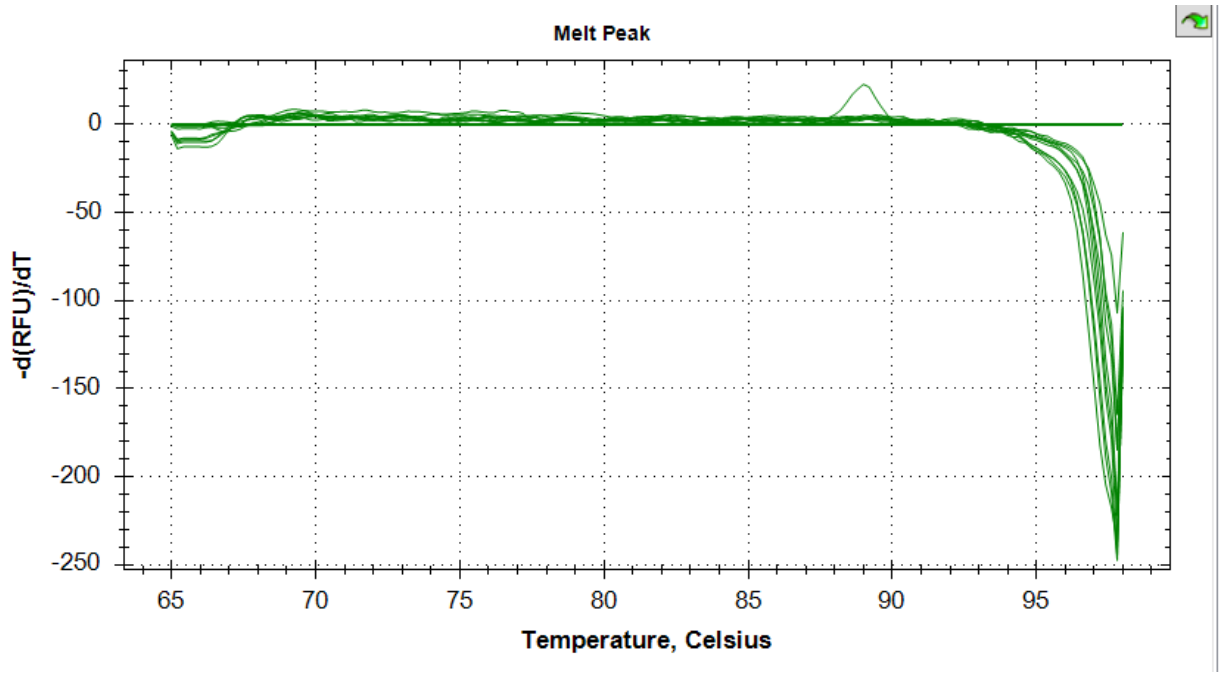


6.2 500 ng RNA in cDNA-reaction; 2 µl cDNA in total concentration of 10 µl

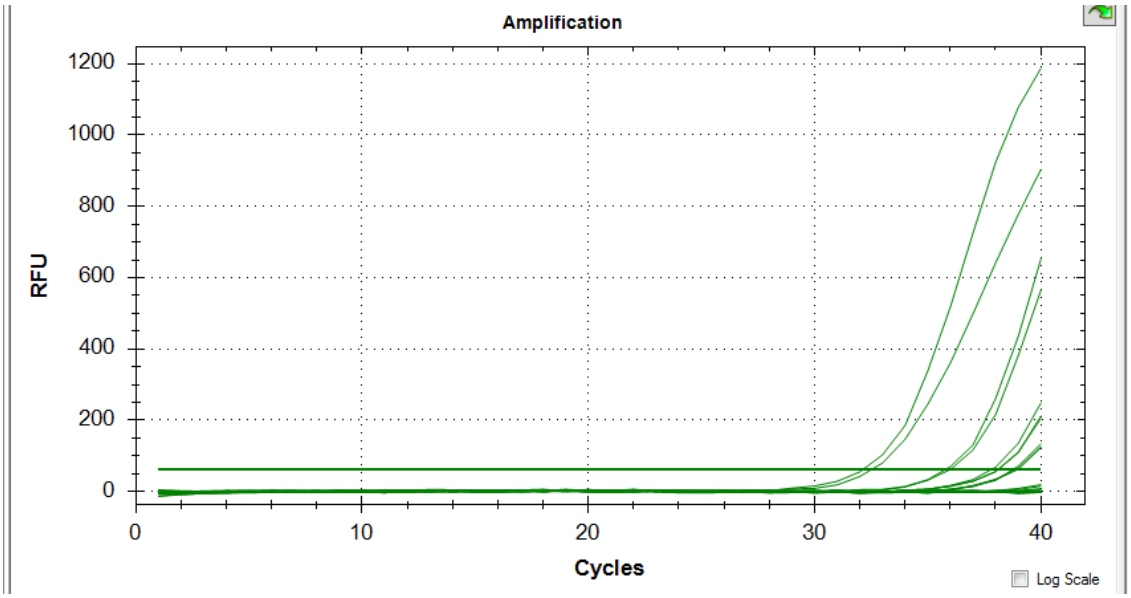


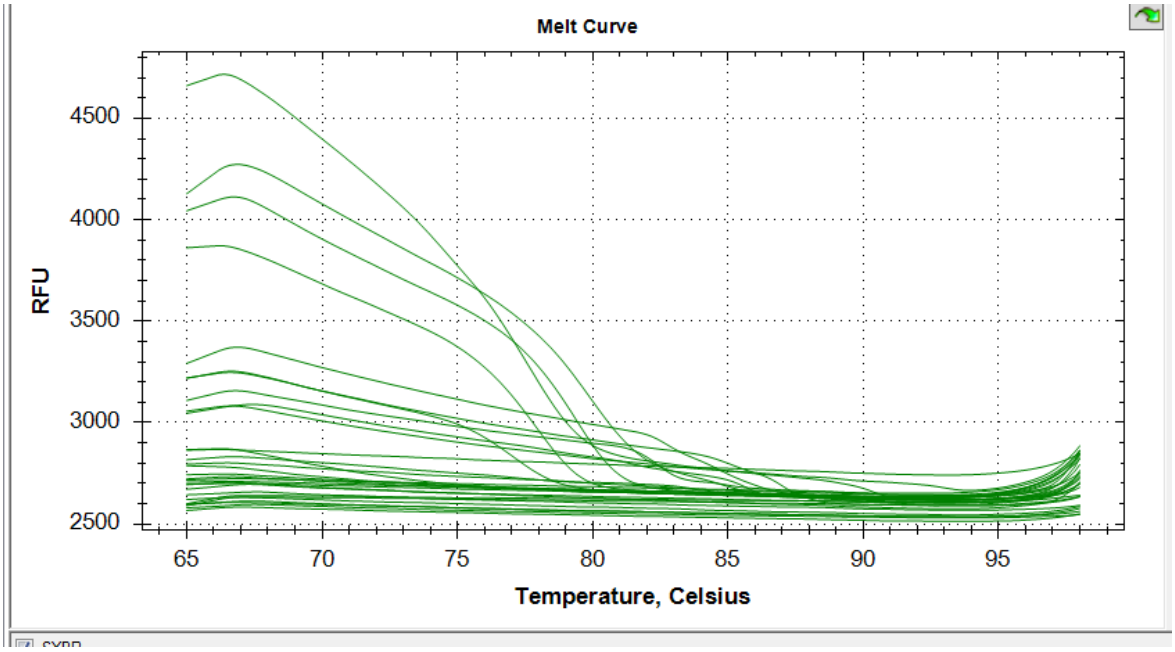



Melt Curve
2900
2800
2700
2600
RFU
65
70
75
80
85
90
95
Temperature, Celsius
SYBR

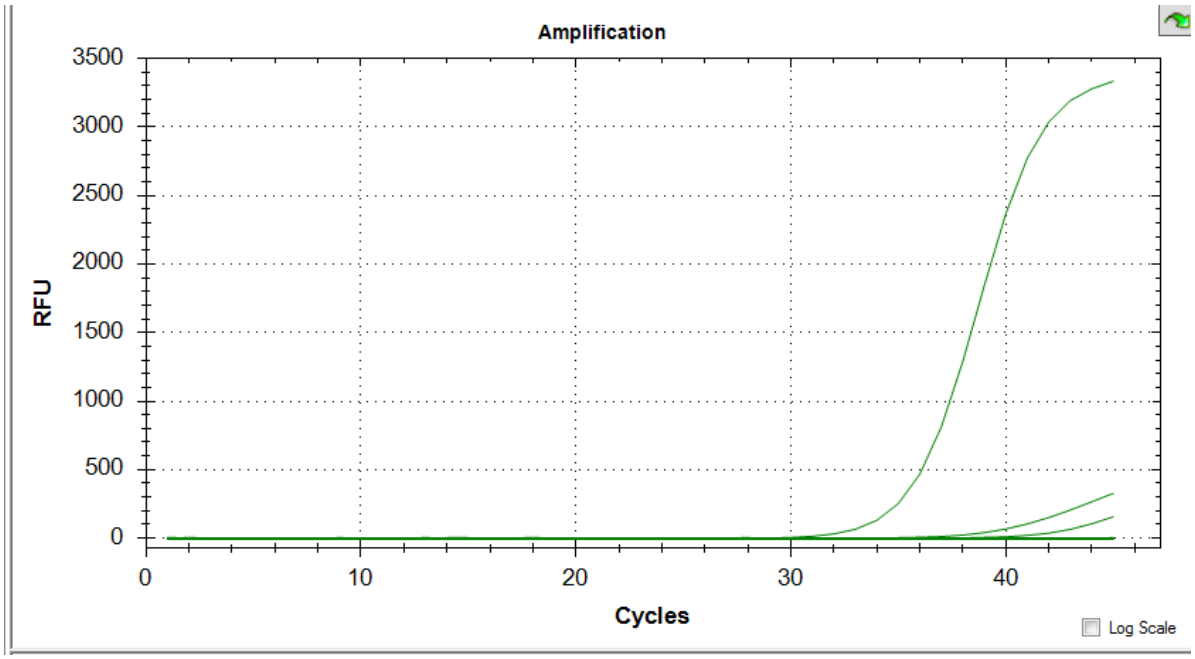

Melt Peak
0
-50
-100
-150
-200
-250
-d(RFU)/dT
65
70
75
80
85
90
95
Temperature, Celsius

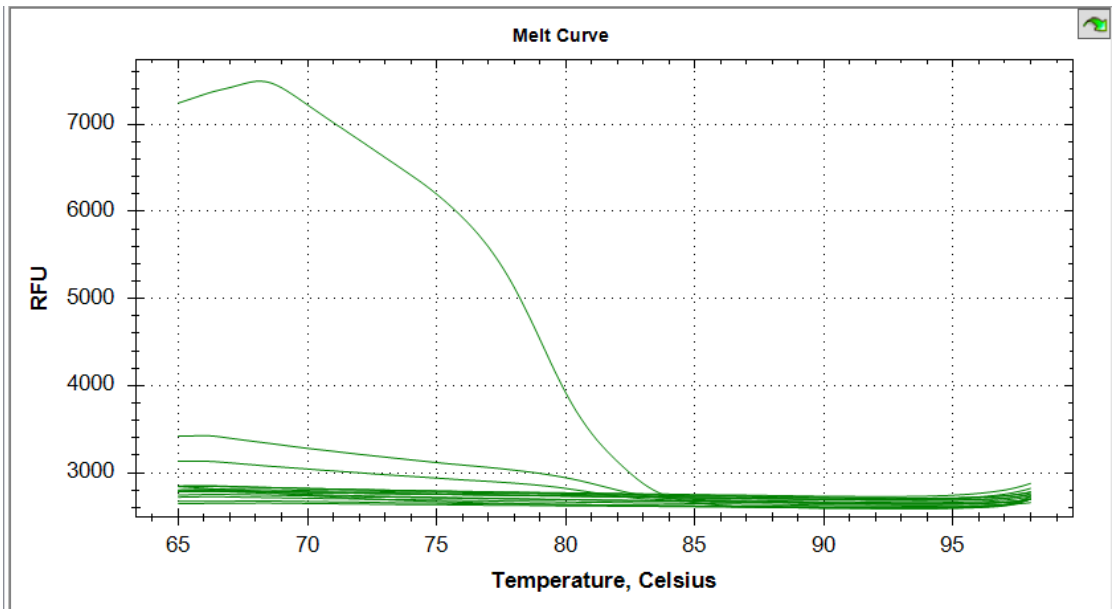
6.3 500 ng RNA in cDNA-reaction; 2 µl cDNA in total concentration of 10 µl



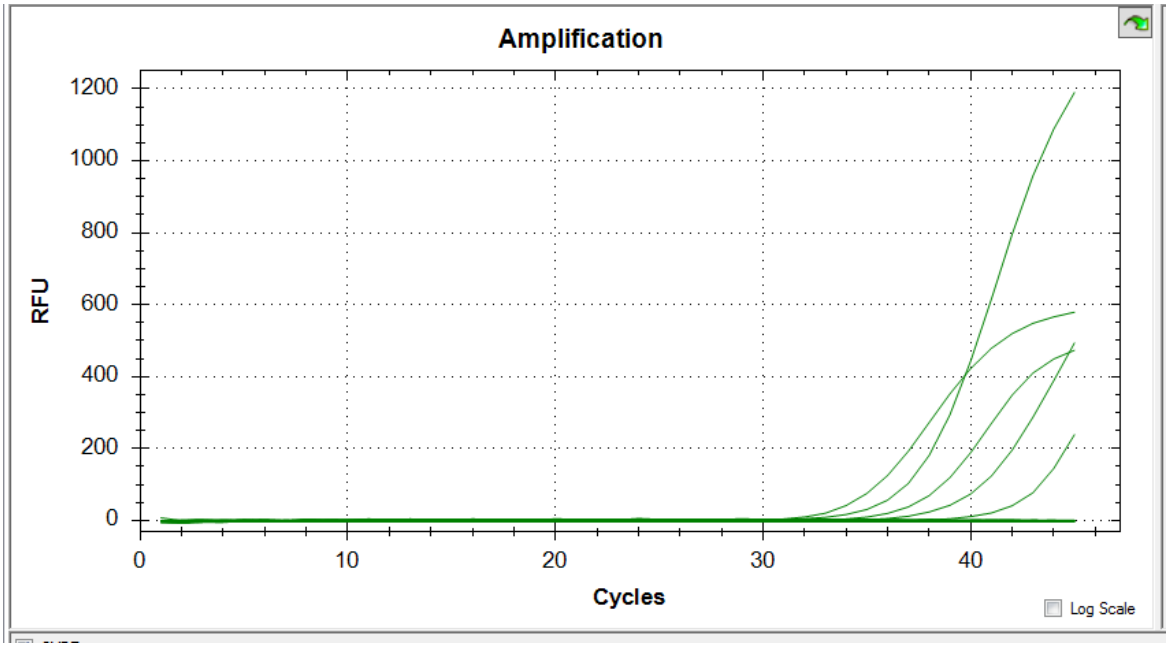


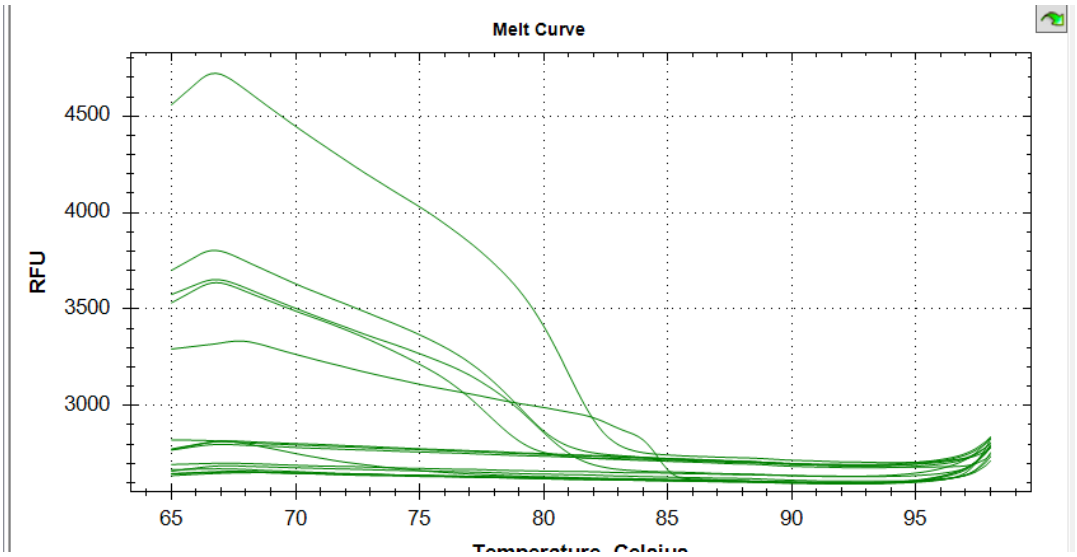
6.4 500 ng of RNA in cDNA-reaction; 7,6 µl cDNA in total concentration of 10 µl





6.5 1000 ng RNA in cDNA-reaction; 2 µl cDNA in total concentration of 10 µl





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