Manual to perform a qPCR with the ABI 7500Fast

Required material:

Product name	REF Nummer / Order No.	costs
MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode	4360954	20 Stk. 183.00 Fr. (Lubio).
MicroAmp® Optical Adhesive Film	4360954	25 Stk. 149.00 Fr. (Lubio)
LightCycler® 480 SYBR Green I Master [1]	04887352001	5ml ca. 200.00 Fr.
Nuclease-free water		
Primer (4.5 µM) [2]		
One channel pipette (10, 100, 1000 µl)		Tips from tick-list
8-channel pipette (10 µl)		Tips from tick-list
Stepper + 0.2 ml tips		Tips from tick-list
Centrifuge in G62 (with plate rotor)		
1.5 ml tubes		Tick-list
qPCR protocol	See next page (pipetting template) – Settings for the protocol are in the following pages (Example of a pipetting template see last page)	

[1]) buy via UTOX – 30% discount on Roche products
[2]) Stock conc. = 100µM ; 4,5µM is „working conc."

Tips:

- Pipette neat and tidy (qPCR is very sensitive)
- Dispense MasterMix with stepper
- First pipette samples, than negative and positive control(s) –and at the end the standards
- Pipette standards with the 8-channel pipette
- If pipetting the same many times, it is worth distributing all the samples in a 96 well plate in advance so you can use a multi-channel pipette
- Stick the adhesive cover foil with Kleenex and not with your gloves or your bare fingers, **foil has to be clean!**
- Pipette the dilutions from A1 to H1 and in duplicate (e.g. in A1 pipette standard 10^7 and in H1 place standard 10^0. The duplicates should be pipetted next to A1 to H1 → A2 to H2. Replicates with the same dilution should be pipetted next to each other, i. e. A1 is duplicated in A2 and not in B1

eawag aquatic research	Aphids quantitative PCR **Lab-Form Primer testing: Analysis**		**Aquatic Ecology**
Created by Marco Thali	created on 17.03.2016	Version 2016.01	*updated : 17.03.2016*

Primer testing with 2x SYBR Green I Master

Reagents	Primer names and/or Aliquot / Lot No	Concentration	Date of preparation/dilution
SYBR Green I Master (Roche)	Product No. 04887352001	2 x	
ddH_2O			
Forward Primer		4.5 µM	
Reverse Primer		4.5 µM	

	Sample names	Concentration	Date / Date of dilution
Sample DNA	see sample sheet file:		see sample sheet from:
		ng/µl	

Procedure:

1. Fill in name of PCR program, annealing temperature, no of PCR cycles, name of PCR machine, no of primer pairs and total no of PCR reactions below

PCR program name	Annealing temp (°C)	Nr of cycles	PCR machine
2-Step		50	Cycler: 11 - ABI 7500Fast

Nr of primer pairs	Total Nr of PCR reactions	Plus volume (%)	Total volume of SYBR mix (µl)
1	96	5	630.00

2. Prepare Mix by adding ddH_2O (I) and 2x SYBR Green I Master (II) in separate tube
3. Add primers (III) / (IV) and mix well
4. Distribute 10 µl of mix into PCR wells
5. Add 2.5 µl of sample DNA or positive/negative control into corresponding PCR well(s)

	Mix per primer		Mix per reaction	Final concentr.
ddH_2O	**126.0** µl	(I)	1.25 µl	
SYBR Green I Master (Roche)	**630.0** µl	(II)	6.25 µl	1 x
Forward Primer	**126.0** µl	(III)	1.25 µl	0.450 µM
Reverse Primer	**126.0** µl	(IV)	1.25 µl	0.450 µM
Total:	1008.0 µl		**10.00** µl	
Sample DNA / controls			**2.50** µl	0 ng/µl
Final volume			12.50 µl	

PCR program 2-Step:

Heated lid	105°C	
50 °C	2 min	
95 °C	10 min	
95 °C	15 sec	50x
0 °C	30 sec	

PCR program mekting curve:

95 °C	15 sec	
60 °C	60 sec	↓30 min
95 °C		
10 °C	∞	

Sample order:

	Primer Fwd												Primer Rev	Species	
A															A
B															B
C															C
D															D
E															E
F															F
G															G
H															H
		1	2	3	4	5	6	7	8	9	10	11	12		

Notes:

Project Group:	Organism(s):	Date:	Person in charge:

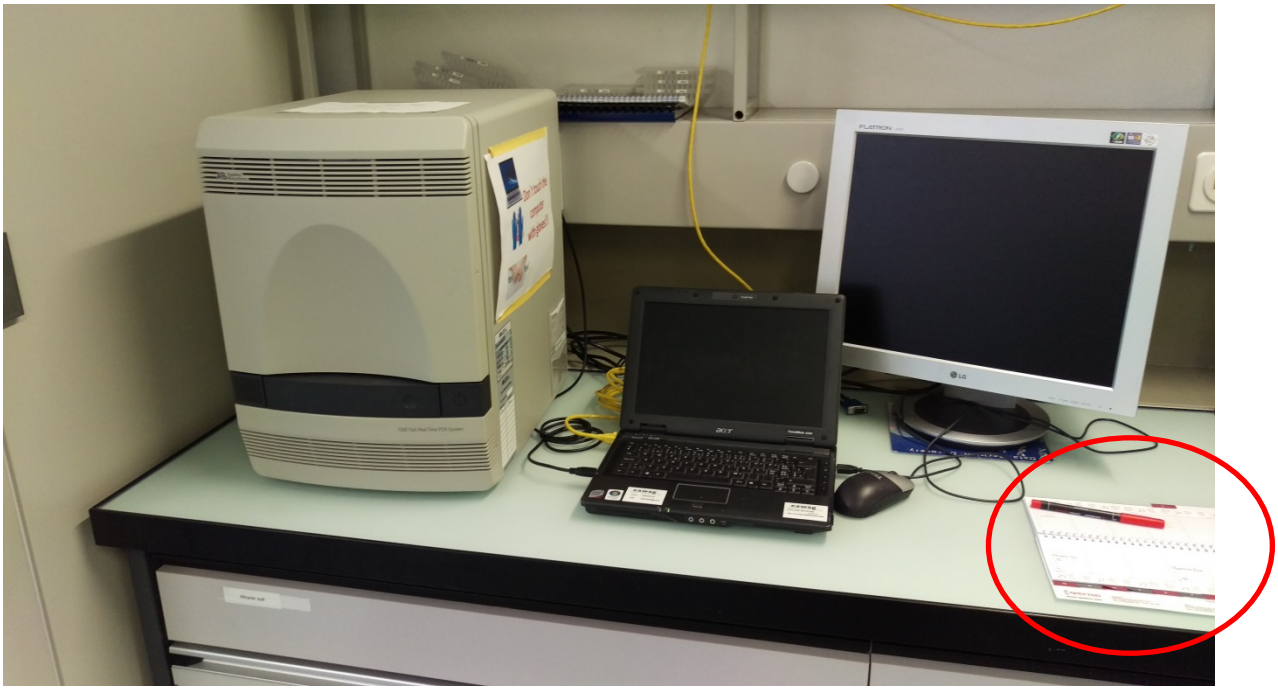
Site of machine: LA-E76 ; right to the -80°C freezer

Person in charge: Smitha Pillai – BU-E09 (Utox) ☎ 5255

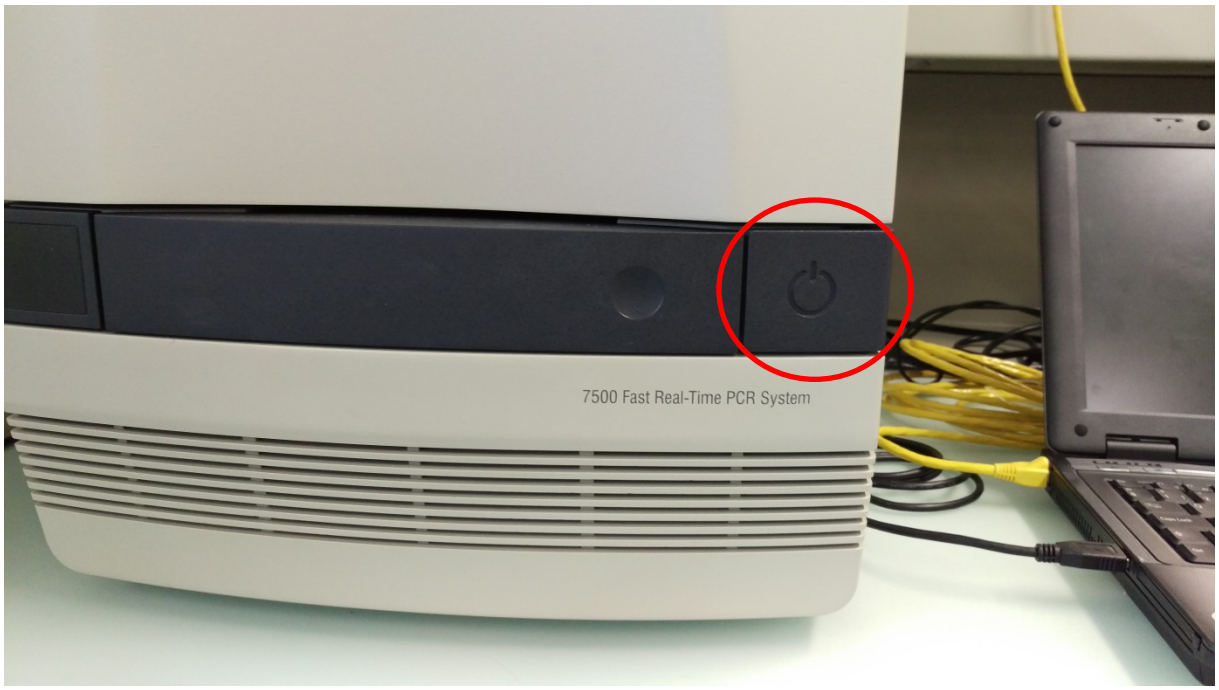
Stephan Fischer – BU-E09 (Utox) ☎ 5567

If you use the cycler for the first time, you have to be introduced by one of the persons in charge!

Book machine in advance with name, time and length of use (calendar is next to the notebook)



Start machine (the best is to start the machine 30 minutes before use to heat up)

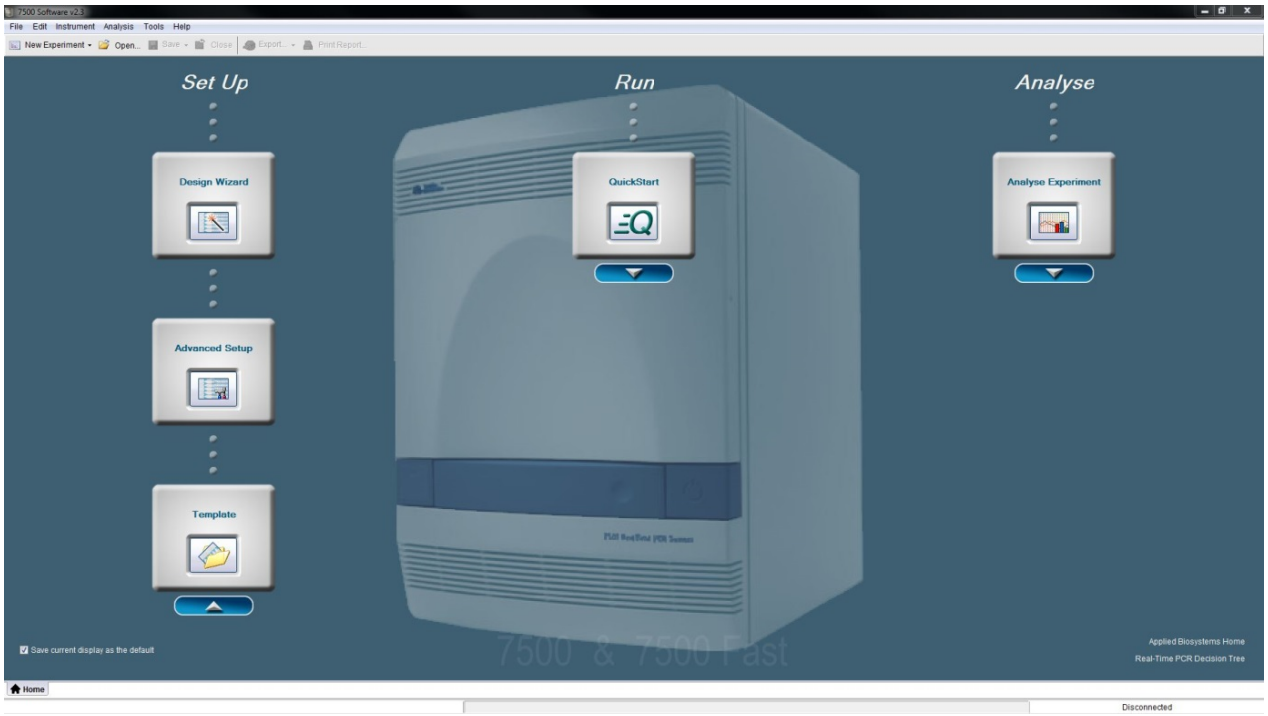


Start computer Username: ABIQPCR (should already be written in)
Password: ABI7500@eaw

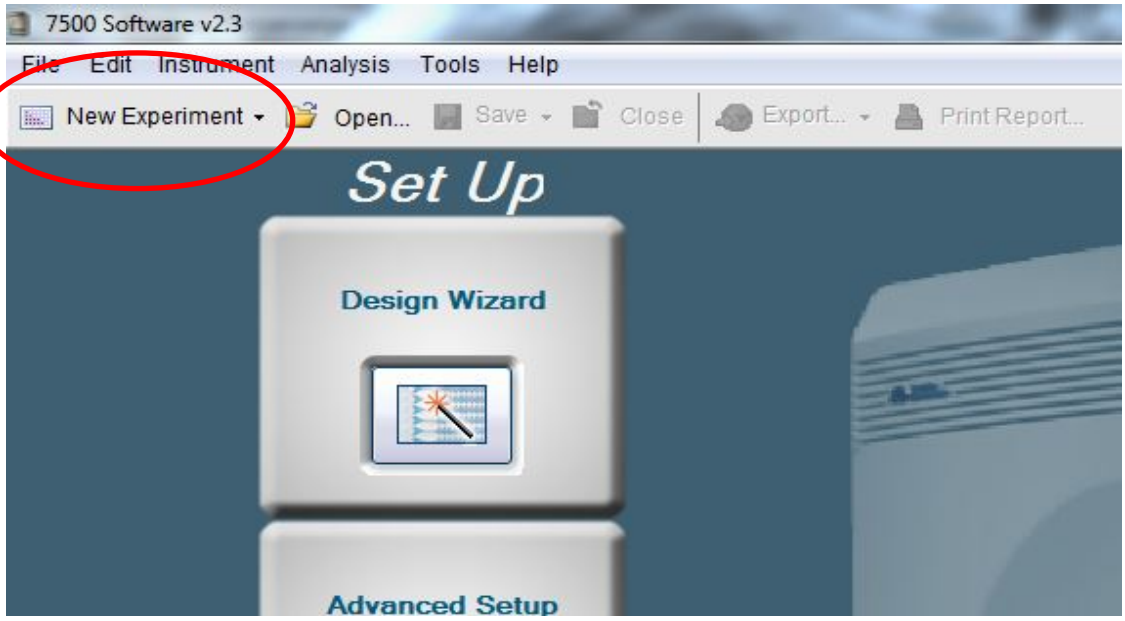
Open software Software name is **7500 Software v2.3**

[If error message appears saying a calibration is required contact Smitha or Stephan]

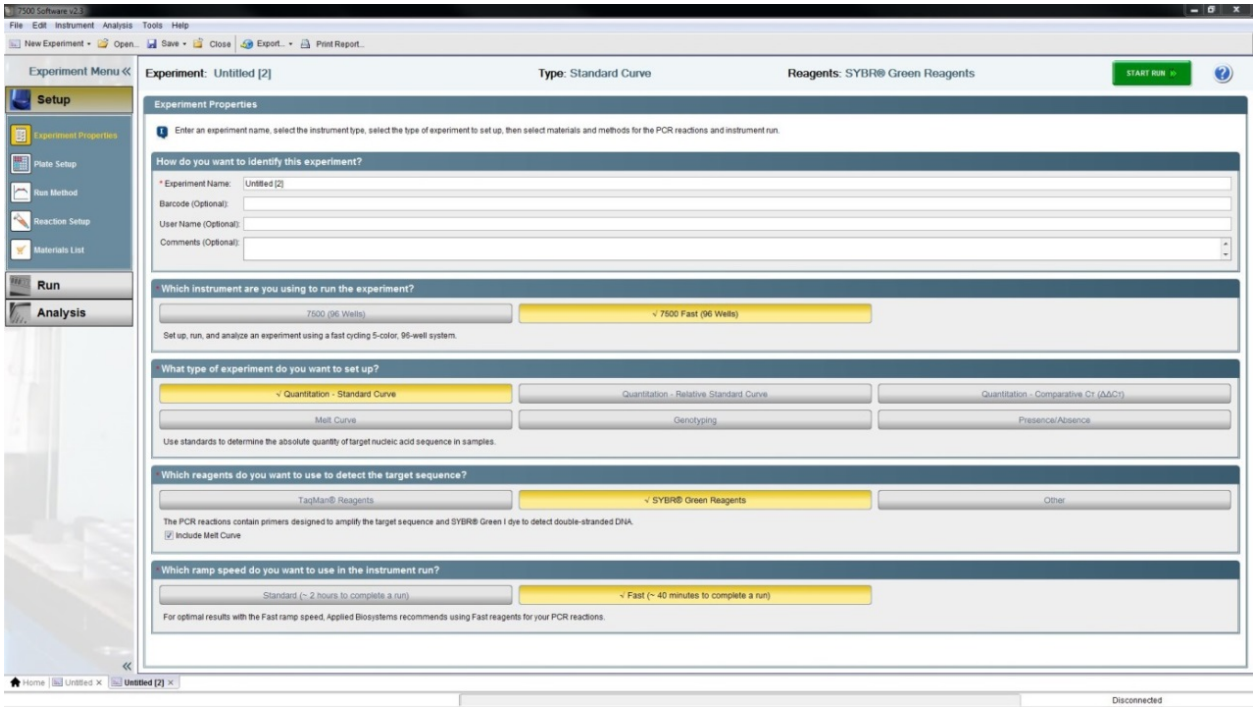
This window should open



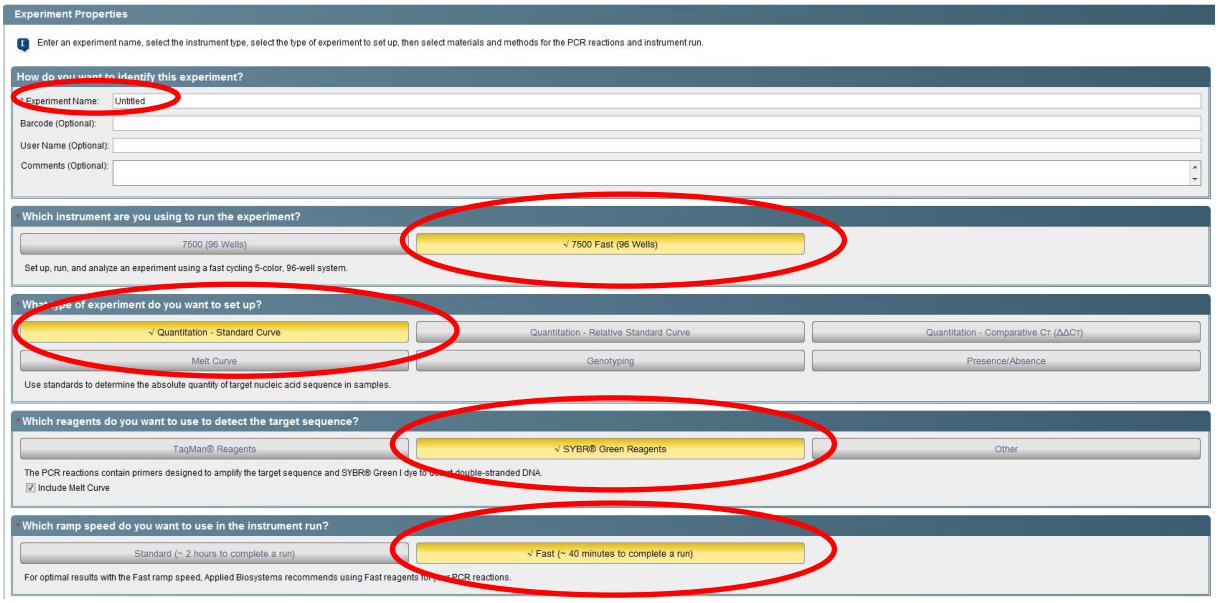
Start new Experiment Click on field **New Experiment** but **not on the arrow**



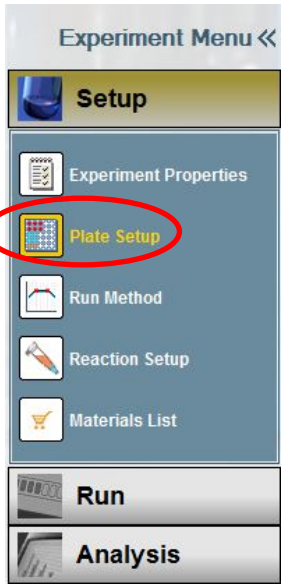
This window should open



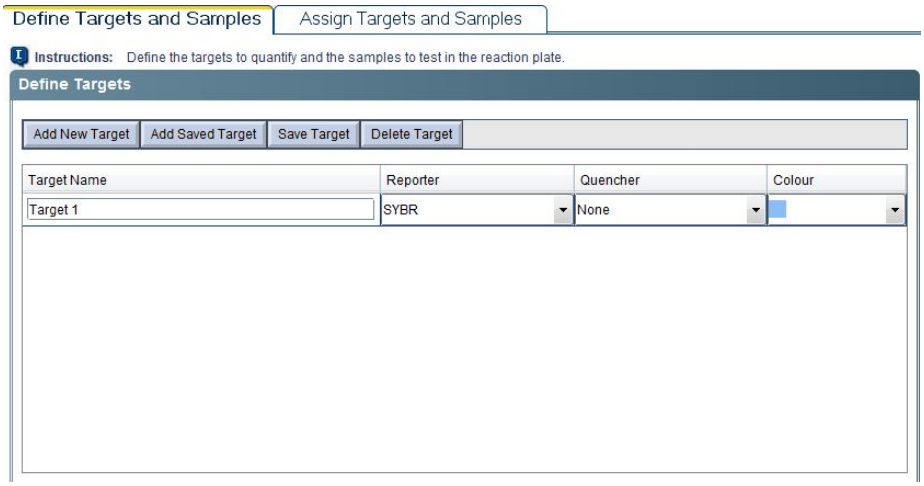
1. Write in experiment name
2. Which Instrument you use: 7500 Fast (96 Wells) If changing the Cycler you must click yes on the message popping up
3. What type of Experiment: Quantitation- Standard Curve
4. Which reagents to detect: SYBR® Green Reagents
5. Which ramp speed: Fast (~ 40 Minutes to complete a run)



Select plate setup (2nd element in the dark blue box left on the screen)

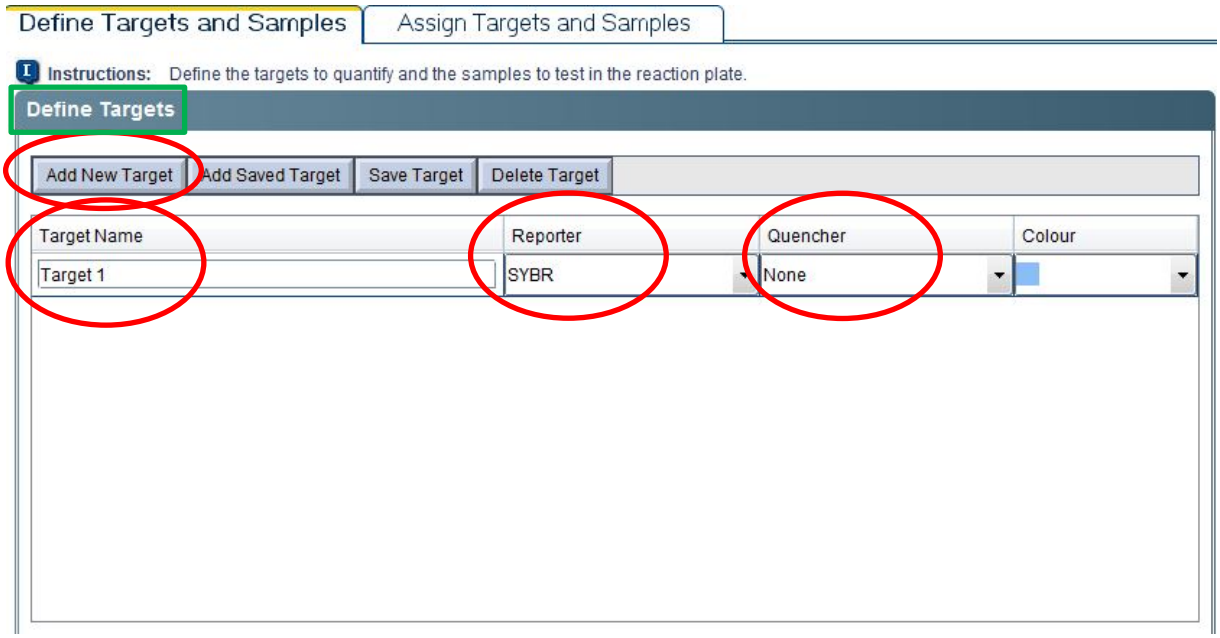


This window get open



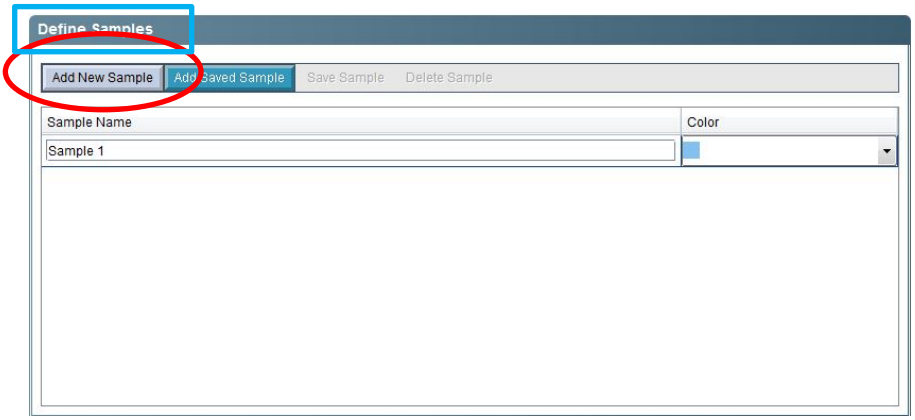
Add New Target

For each gene amplified you have to create a new target. Then fill in Target Name, Reporter is SYBR and Quencher is none (Target one is filled in automatically)



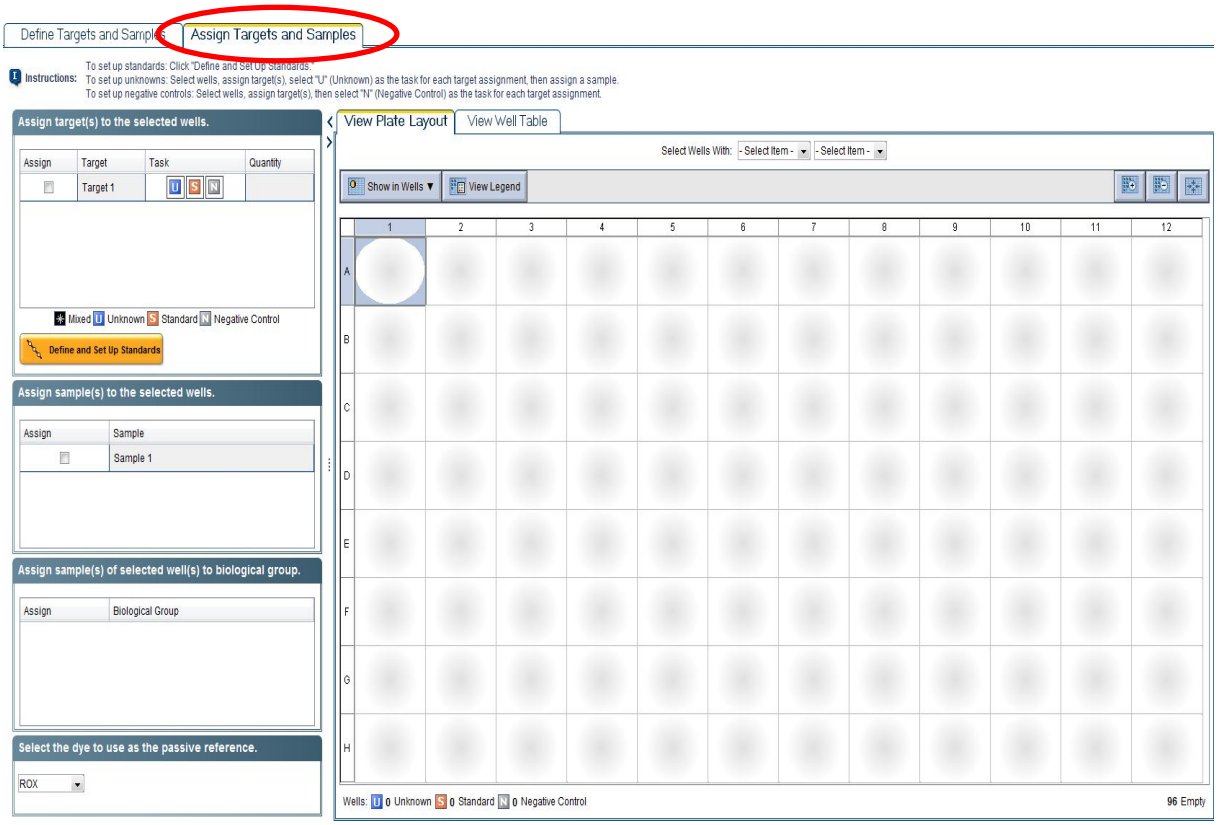
Define Samples:

Therefore select **add new sample** and fill in names (find on the right of "define targets)

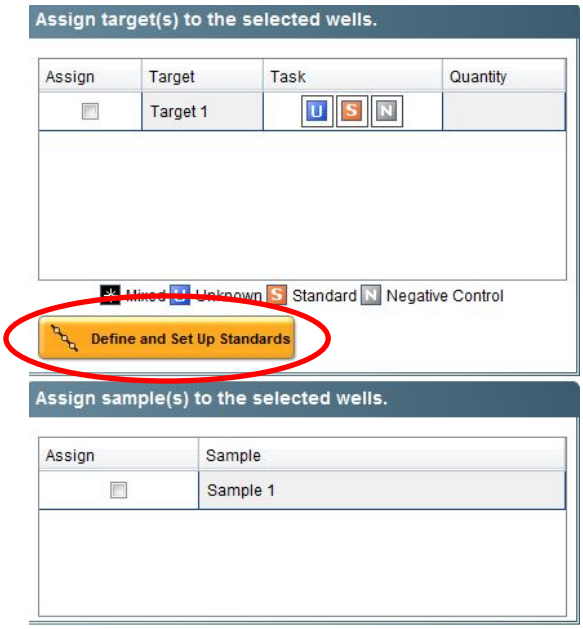


Here, all samples (incl. standards and controls) should be assigned. So for each used „well" on the plate, a sample must be defined.

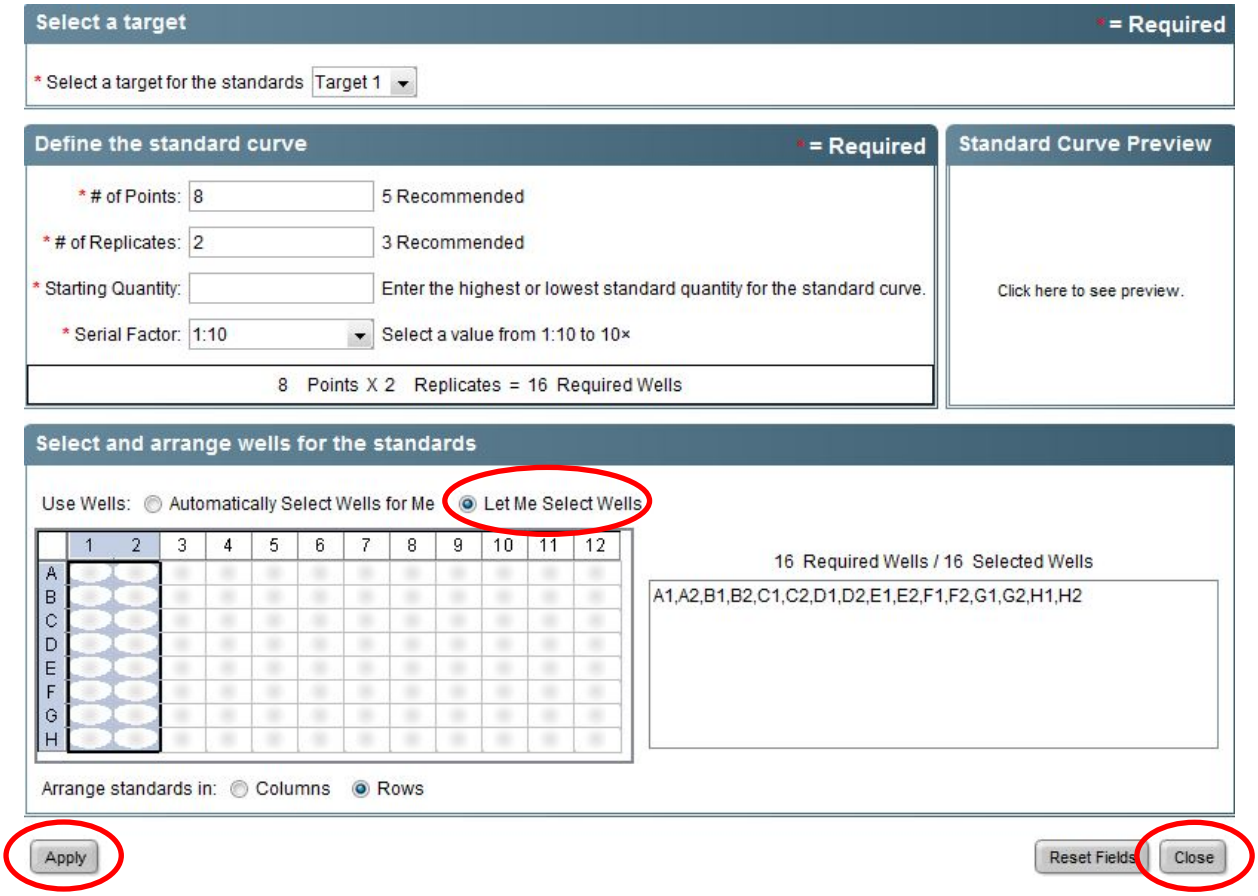
Select Assign Targets and Samples: Each well must be assigned to the corresponding sample



First the standard curve must be defined Click **Define and Set Up Standards**



The following window opens



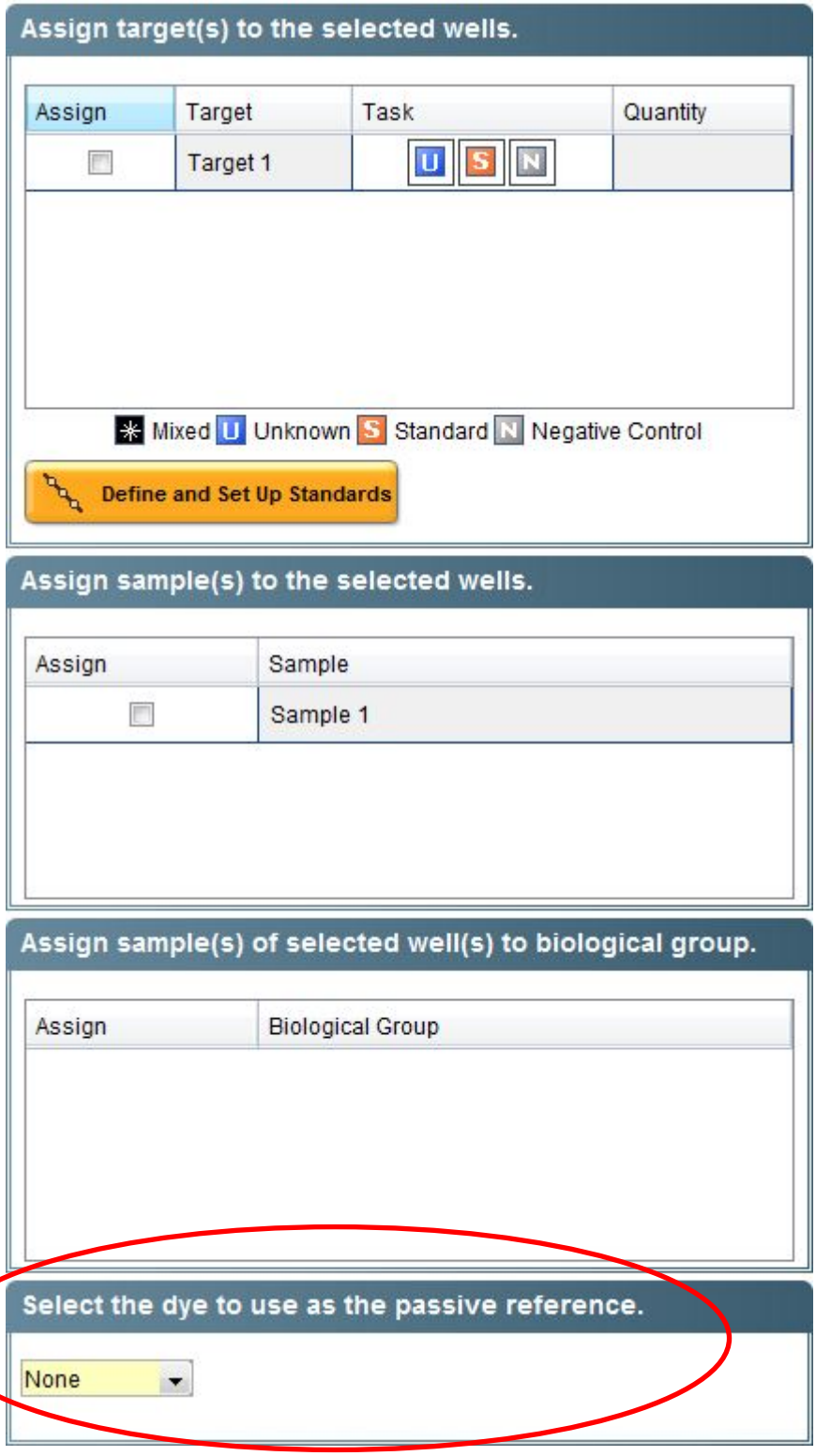
Select Target:	Select respective target
# of Points:	Number of dilutions you made
# of Replicates:	Number of replicates for each dilution
Starting Quantity:	Highest standard concentration (e.g. 100'000)
Serial Factor:	select a dilution factor (1:2 up to 1:10 are possible)

Select **Let Me Select Wells**, then mark the desired wells. Afterward select **Apply** (if field "close" is selected before selecting apply, the standard curve is not saved and you have to do everything again)

Select **Close** to close the windows

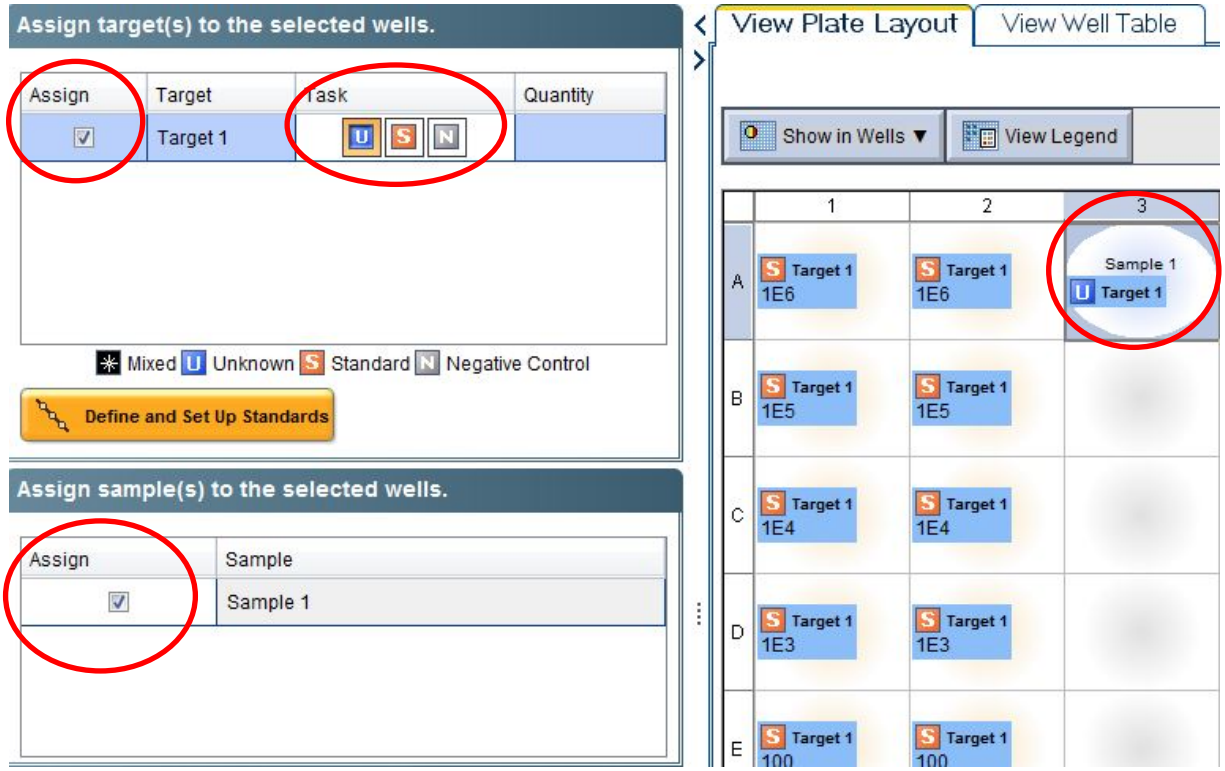
Afterward define the passive-reference:

Make sure that in **Select the Dye to use as the passive Reference** you mark **„None“**
(This setting is below the field where the standards can be defined)



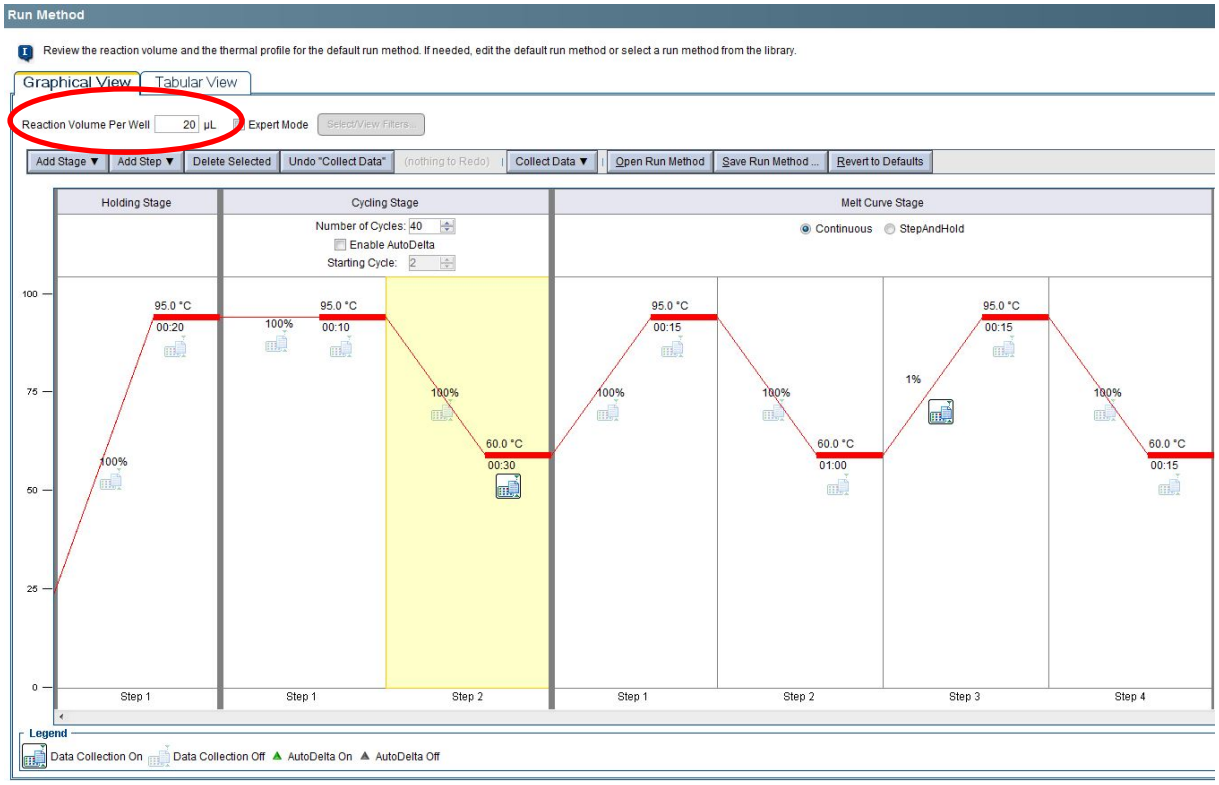
After that, all samples will be defined:

Define Samples:
- Mark desired well on the plate
- Set a “check mark” for the right target in the “Assign”-column
- Choose if the sample is unknown(U) or a negative control (N)
- Set a “check mark” to choose the right sample



Select run method (3rd element in the dark blue box left of the screen)

Fill in the right reaction volume



Add Stage and Steps:

Add Stage: Here it is possible to add a new stage (holding and cycling stages are possible). The melt curve stage should be in the run method automatically, if not then control if TaqMan® is selected in **which reagents to detect**. If there is not **SYBR® Green** marked, no melt curve is possible.

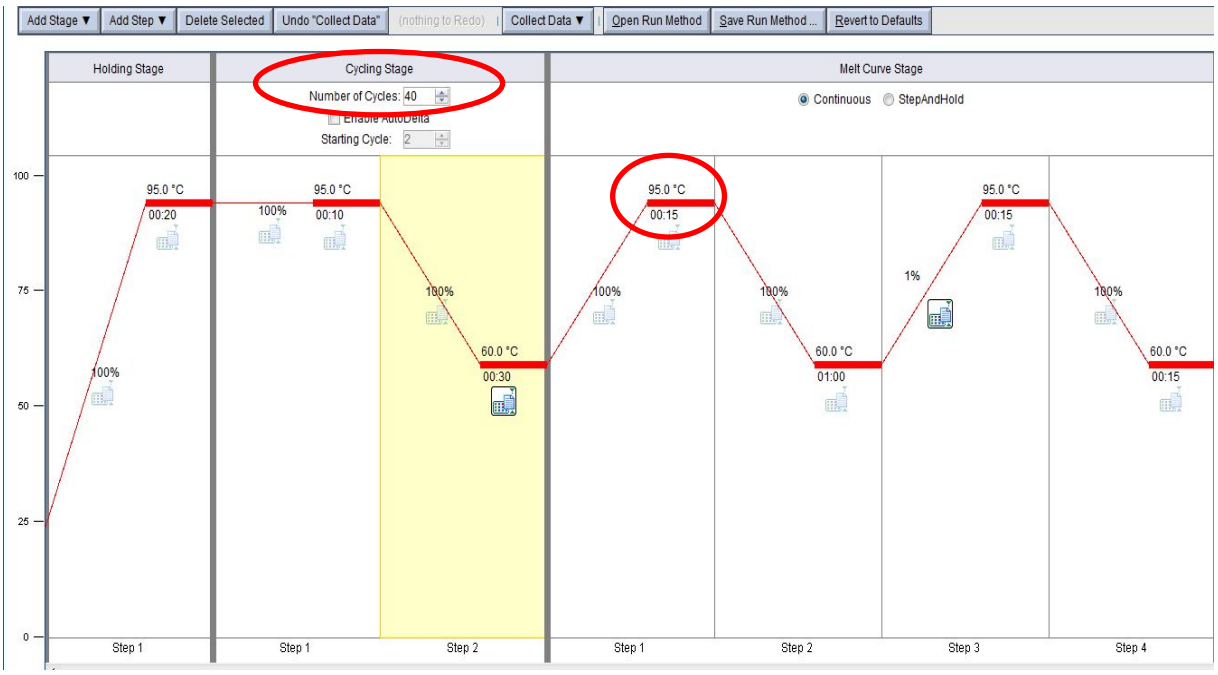
Add Step: Define if the step should be added before or after the marked step (yellow)

Delete Selected: The yellow marked stage or step will be deleted

Fill in the right values for temperature and time (cycling conditions)

Fill in the cycle number

This sign  stands for the measuring point (must be in the elongation step in the cycling stage and in the melt curve stage it should be set duringthe increasing of the temperature). If a yellow field appears called **The number of data collection points is not valid**, then you must control if there is a measuring point set somewhere wrong. This can be seen through controlling if the sign is slightly transparent (point inactive) or not (point active)



1% slope in the melt curve means, that the temperature will be increased from 65°C to 95°C within ~30 minutes.

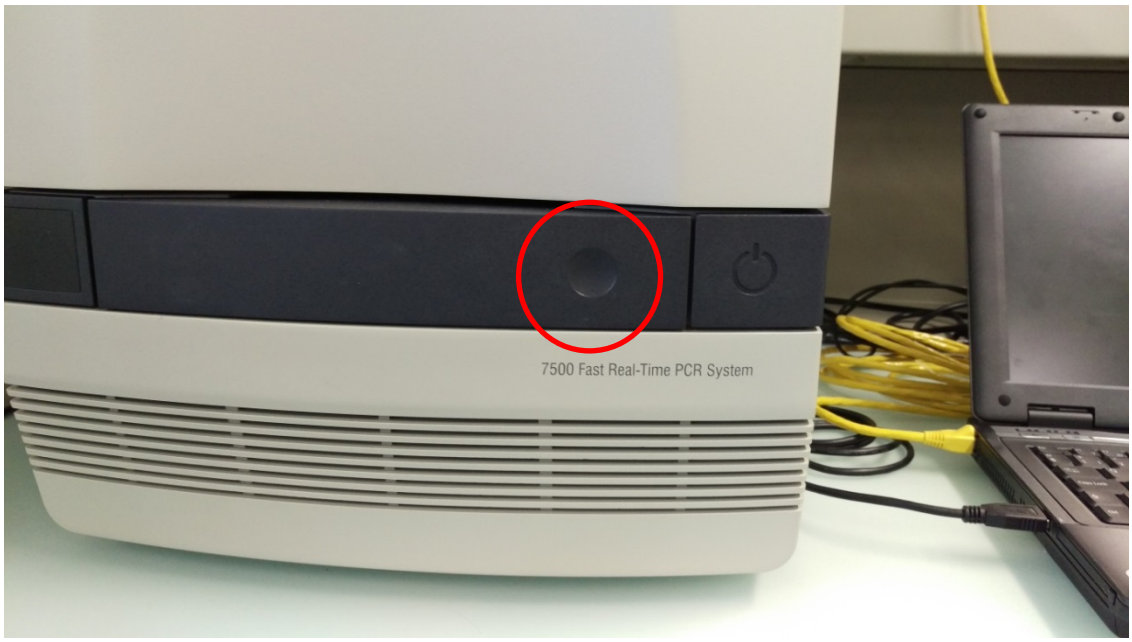
A **Holding stage** always consists of **1 step (hold of temperature)**

A **Cycling stage** always consists of **2 steps (denaturation + annealing/elongation)**

A **Melt curve stage** always consists of **4 steps (4x hold temperature)**

Put plate in ABI7500:

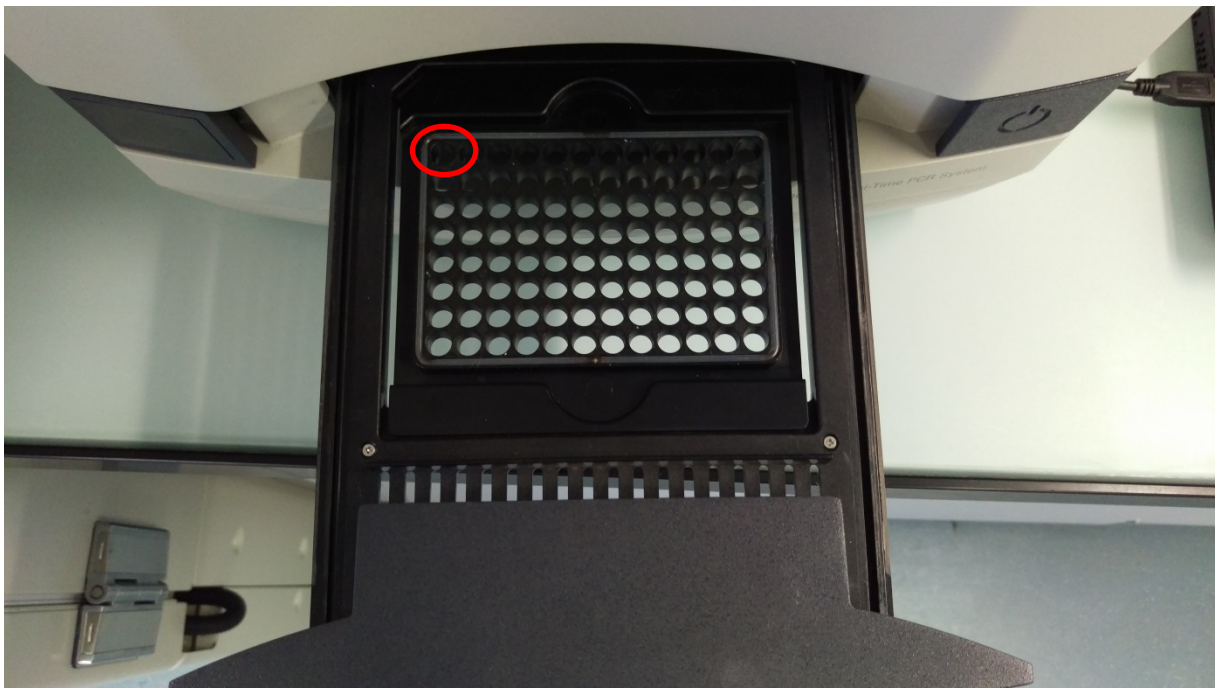
In the recess of the tray press firmly until it „clicks“ so that the tray comes out by **itself**



Put plate in ABI cycler

Well A1 must be on top left!

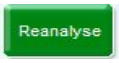
Then press the tray back (press firmly again) until it „clicks“ again



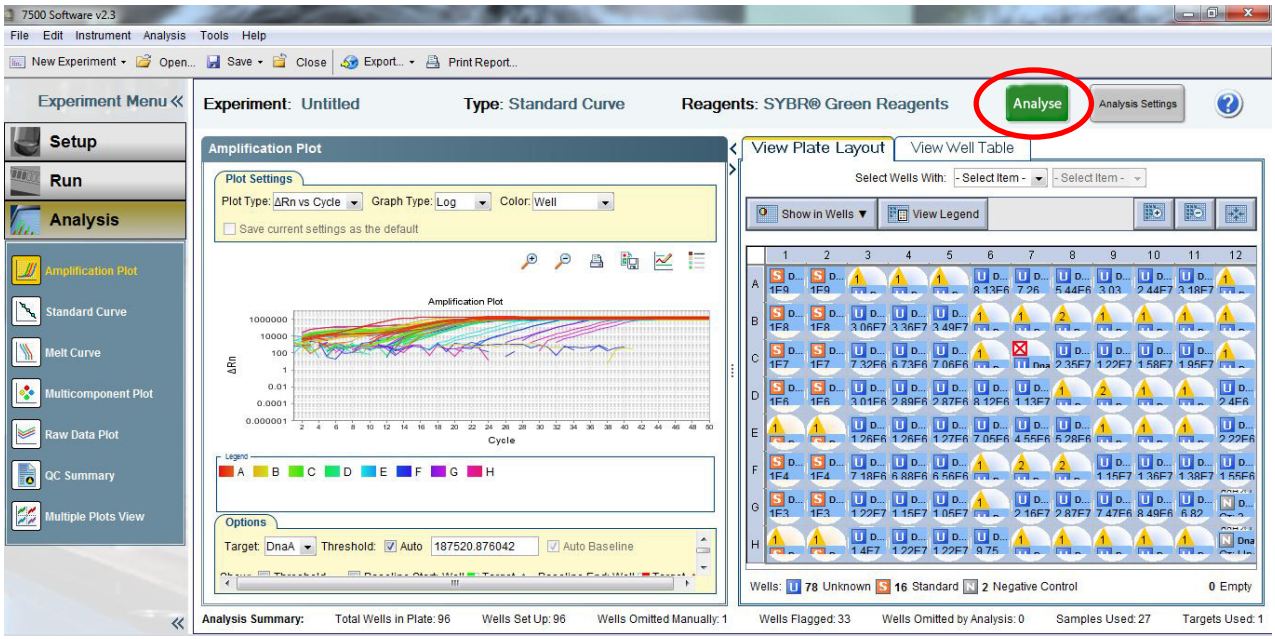
When everything is correct, press 

Important: **Stay near the Cycler until the first or second cycle is finished. Sometimes an error message appears that the Cycler has a problem and does not start!**

After the run's finished:

To receive data press the green button **ANALYSE** (top right). After that it says 

on the button. This way you can check, if you have pressed "analyse" already in case you are not sure anymore.

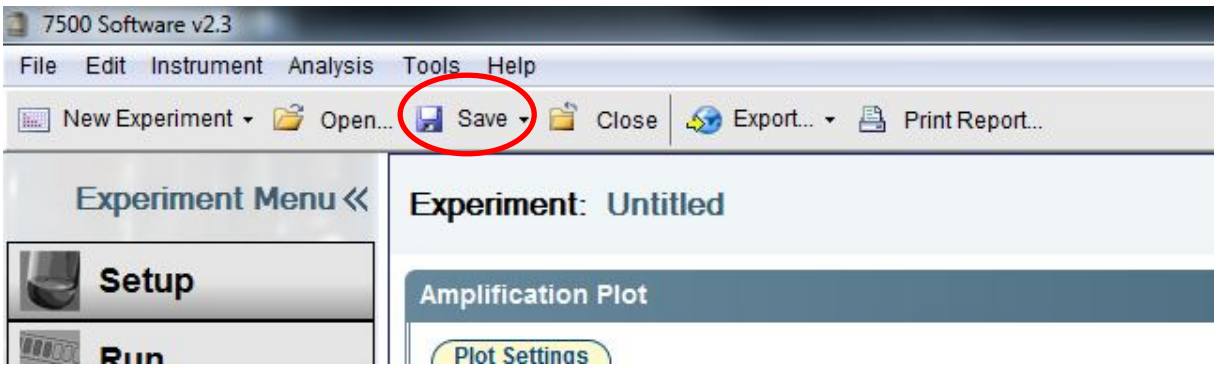


Save run:

Press on the arrow next to the diskette symbol and then on **save as** to select the save location.

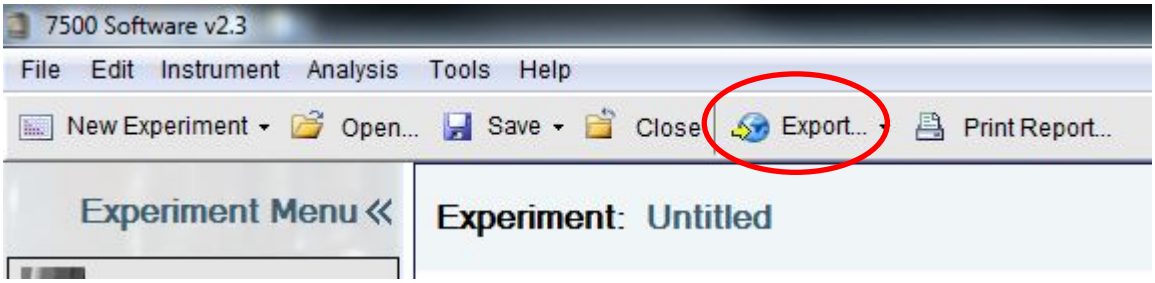
If repeating the same experiment more than once, it can be saved as template.

Never save your run as a template before you've selected "Save As". If you do, your data will be lost!!!

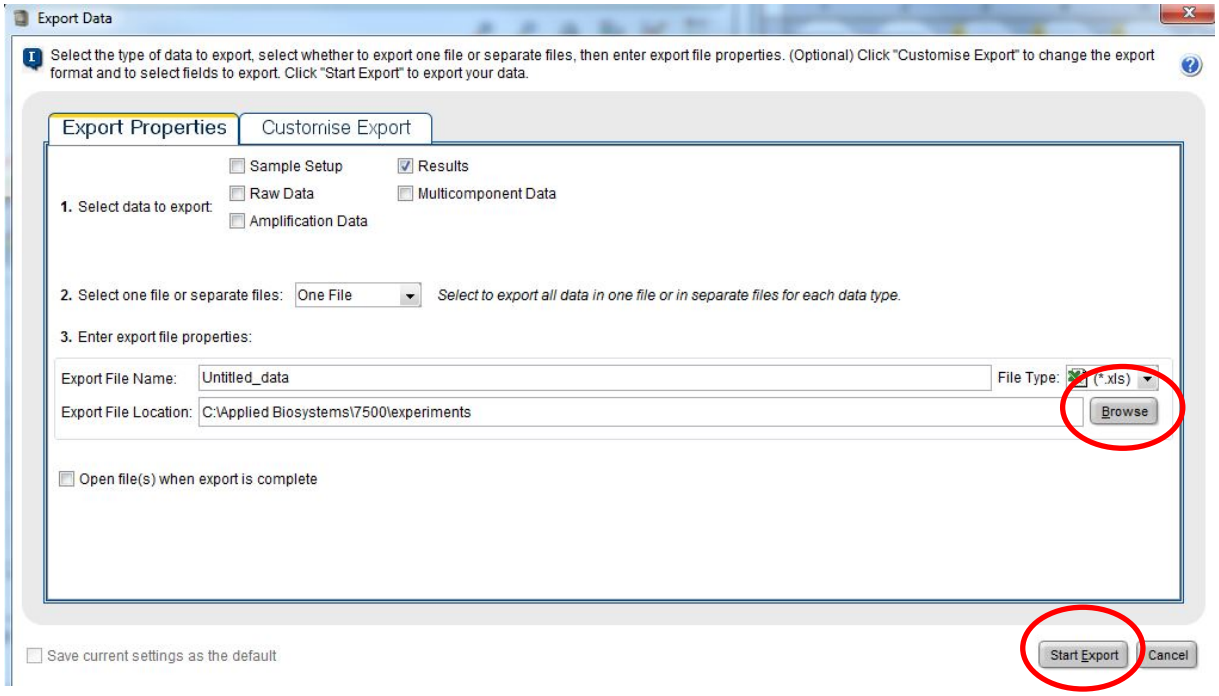


Export data:

Press on **Export**



This window should open



In 1. select which data you want to export (important: make sure you check “results”)

If you select more than one checkbox in 1. Then in 2. You can choose if all the data should be exported in one file or in separate files (samples setup, raw date, etc.).

In 3. choose a file name and a saving location (press browse).

Then press **Start Export**

After exporting all the data a window pops up asking whether you want to export more data or close the window. This window can be closed.

Now remove plate from cycler and throw away if not needed anymore!

At the end turn off the Cycler and the notebook.

Example of a qPCR pipette template

eawag aquatic research	Aphids quantitative PCR **Lab-Form Primer testing: Analysis**		**Aquatic Ecology**
Created by Marco Thali	created on 17.03.2016	Version 2016.01	*updated : 17.03.2016*

Primer testing with 2x SYBR Green I Master

Reagents	Primer names and/or Aliquot / Lot No	Concentration	Date of preparation/dilution
SYBR Green I Master (Roche)	Product No. 04887352001	2 x	
Ap_DnaA_Fwd	5'-AAT GCT TGG ATC ATA ATT TAA AGA C-3' - 2558179	4.5 µM	05.04.2016
Ap_DnaA_Rev	5'-GTT TTG AAG AAA GAA ATG TTT CAA G-3' - 2558180	4.5 µM	05.04.2016
Ap_EF1a_Fwd	5'-TAG CAG TTA CAT CAA GAA AAT CGG-3' - 2558177	4.5 µM	05.04.2016
Ap_EF1a_Rev	5'-ATG TTG TCT CCA TTC CAT CCA G-3' - 2558178	4.5 µM	05.04.2016

	Sample names	Concentration	Date / Date of dilution
Sample DNA	see sample sheet file:		see sample sheet from:
		ng/µl	05.04.2016

Procedure:

1. Fill in name of PCR program, annealing temperature, no of PCR cycles, name of PCR machine, no of primer pairs and total no of PCR reactions below

PCR program name	Annealing temp (°C)	Nr of cycles	PCR machine
2-Step	57	50	Cycler: 11 - ABI 7500Fast

Nr of primer pairs	Total Nr of PCR reactions	Plus volume (%)	Total volume of SYBR mix (µl)
1	96	5	630.00

2. Prepare Mix by adding ddH_2O (I) and 2x SYBR Green I Master (II) in separate tube
3. Add primers (III) / (IV) and Probe (V) and mix well
4. Distribute 10 µl of mix into PCR wells
5. Add 2.5 µl of sample DNA or positive/negative control into corresponding PCR well(s)

	Mix per primer		Mix per reaction	Final concentr.
ddH_2O	**126.0** µl	(I)	1.25 µl	
SYBR Green I Master (Roche)	**630.0** µl	(II)	6.25 µl	1 x
Forward Primer	**126.0** µl	(III)	1.25 µl	0.450 µM
Reverse Primer	**126.0** µl	(IV)	1.25 µl	0.450 µM
Total:	882.0 µl		**10.00** µl	
Sample DNA / controls			**2.50** µl	0 ng/µl
Final volume			12.50 µl	

PCR program 2-Step:

Heated lid	105°C	
50 °C	2 min	
95 °C	10 min	
95 °C	15 sec	50x
57 °C	30 sec	

PCR program mekting curve:

95 °C	15 sec	
60 °C	60 sec	↓ 15 min
95 °C		
10 °C	∞	

Sample order:

	Primer Fwd	Standardreihe		Proben									Primer Rev	Species	
A	*Ap_DnaA_Fwd*	10^9	10^9	A15-10	A15-10	A15-10	A15-383	A15-383					*Ap_DnaA_Rev*	*Spiroplasma*	A
B		10^8	10^8	A15-11	A15-11	A15-11	A15-385pi	A15-385pi							B
C		10^7	10^7	A15-17	A15-17	A15-17	A15-392gr	A15-392gr							C
D		10^6	10^6	A15-27	A15-27	A15-27	OX-C161	OX-C161							D
E		10^5	10^5	A15-198bis	A15-198bis	A15-198bis	A15-383	ddH2O							E
F		10^4	10^4	A15-316	A15-316	A15-316	A15-385pi	ddH2O							F
G		10^3	10^3	A15-364	A15-364	A15-364	A15-392gr								G
H		10^2	10^2	A15-370	A15-370	A15-370	OX-C161								H
		1	2	3	4	5	6	7	8	9	10	11	12		

Notes:

Project Group:	Organism(s):	Date:	Person in charge:
	Spiroplasma sp.		