STORE

SOP Title:

Quantitative reverse transcription real-time PCR with mRNA from fresh-frozen or formalin-fixed paraffin embedded tissue

The STORE processing methods were shown to be fit-for purpose for DNA, RNA and protein extraction from FFPE material. The STORE characterisation methods were shown to be fit for-purpose for Quality Control of the extracted DNA, RNA and proteins.

HUMAN CANCER STUDIES GROUP

SOP reference MBXX

Standard Operating Procedure for

Quantitative reverse transcription real-time PCR with mRNA from fresh-frozen or formalin-fixed paraffin embedded tissue

Version number ……1……

Date created ……28/09/2010

Date of review ……28/09/2011……

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Summary
This protocol explains the procedure measuring mRNA expression of genes using quantitative reverse-transcription PCR.

Safety Considerations

Protective clothing and gloves should be worn throughout.

Introduction

The following protocol describes analysis of single gene mRNA expression with RNA from fresh-frozen or from FFPE tissue sections using the Taqman (Applied Biosystems) chemistry. The advantage of using Taqman is the short length of amplimers which typically ranges from 60bp to 120bp what is smaller than the smallest intact fraction of mRNA from FFPE tissue (> 200bp) what makes Taqman well usable for mRNA expression analysis in combination with FFPE tissue. It has been shown by us and by others that qRTPCR using small amplimers is well applicable on mRNA extracted from FFPE tissues. This is only limited by an overall reduction of the relative amount of the intact fraction of transcripts in RNA extracted from FFPE and therefore reduces the number of low expressed transcripts that can be analysed.

Method

Selection of probes

Probes and primers can be selected from the Applied Biosystems website (access through ICIS).

Browse to the section 'Taqman Gene Expression Assays' and tick “H. sapiens” under 'Choose Species'. Fill in the gene symbol (e.g. Pten) in the search field and wait for search results. The search comes up with Assay Ids – with a prefix and a suffix . Clicking the '?' lets pop up a window explaining all different types of probes. You are looking for probes with the prefix “Hs_” (human) and the suffix “_m1” (exon-spanning – no detection of the gene in genomic DNA). All probes with best performance and shortest amplimer lengths are marked with a * - choose if possible this probe. If there is no probe recommended go for the shortest amplimer size and for an “inventoried” probe. There are “inventoried” and “made to order” probes. The latter are, triggered by your order, tested and quality tested and it takes 4-8 weeks for the probe to be delivered whereas made-to order assays already passed this process and are delivered within one week after you ordered them.

Storage of assays

The assays (~ 250 µl) are stored in the -20 C freezer. Transfer the solution from the pipetting robot tubes which are not handy into a 1.5 ml tube when you use it the first time and label the tubes with the gene symbol and the assay Id. Split the assay into multiple aliquots in order to avoid too many freeze-thaw cycles.

Planning of your experiment

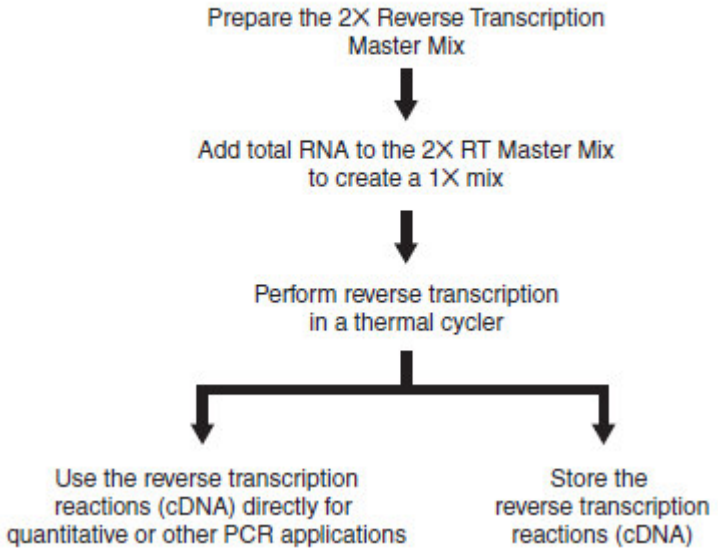
The real-time PCR cycler (ABI 7500) which is based at the MRC Microarray Centre (7th floor Commonwealth Building) has to be booked before using it. Put your name and extension number into the book beside the PCR cycler and choose one or more of available time slots. If you cannot use a slot although booked cancel your booking and inform following users when you are delayed.

Estimated of setting up plate (cDNA ready): ~ 1h
Length of PCR run: 1h30min

Reverse-transcription

Overview

To synthesize single-stranded cDNA from total RNA using the High Capacity cDNA Reverse Transcription Kits:



Material

- High Capacity cDNA Reverse Transcription Kit, 200 reactions (part nr.: 4368814, Applied Biosystems)
- 0.2 µl PCR tubes (single or 8-stripes)
- PCR cycler (GeneAmp 9700)
- 0.5 – 1 µg total RNA per sample

Protocol

1. label one 0.2 µl tube per sample with sample name and put on the metal plate on ice

2. Referring to the table below, calculate the volume of components needed to prepare the required number of reactions.

   Note: Prepare the RT master mix on ice.

Component	Volume/Reaction (µL)	
	Kit with RNase Inhibitor	Kit without RNase Inhibitor
10X RT Buffer	2.0	2.0
25X dNTP Mix (100 mM)	0.8	0.8
10X RT Random Primers	2.0	2.0
MultiScribe™ Reverse Transcriptase	1.0	1.0
RNase Inhibitor	1.0	—
Nuclease-free H_2O	3.2	4.2
Total per Reaction	10.0	10.0

   IMPORTANT! Include additional reactions in the calculations to provide excess volume for the loss that occurs during reagent transfers.

   WARNING **CHEMICAL HAZARD. 10× Reverse Transcription Buffer** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate eyewear, clothing, and gloves.

3. Place the 2X RT master mix on ice and mix gently.

To prepare the cDNA RT reactions:

1. Pipette 10 µL of 2X RT master mix into each well of a 96-well reaction plate or individual tube.
2. Pipette 10 µL of RNA sample into each well, pipetting up and down two times to mix.
3. Seal the plates or tubes.
4. Briefly centrifuge the plate or tubes to spin down the contents and to eliminate any air bubbles.
5. Place the plate or tubes on ice until you are ready to load the thermal cycler.

To perform reverse transcription:

1. Program the thermal cycler conditions using one of the thermal cyclers listed in Table 3 on page 4.

   IMPORTANT! These conditions are optimized for use with the High Capacity cDNA Reverse Transcription Kits.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

2. Set the reaction volume to **20 µL**.
3. Load the reactions into the thermal cycler.
4. Start the reverse transcription run.

Storage

You can store cDNA RT plates or tubes prepared using the High Capacity cDNA Reverse Transcription Kits for short-term or long-term storage.

Storage Duration	Storage Temperature (°C)
Short-term (up to 24 hours before use)‡	2 to 6
Long-term	–15 to –25

Real-Time PCR

Material

- MicroAmp Fast 96-Well Reaction Plates, 0.1 ml (part-nr.: 4349607, Applied Biosystems)
- MicroAmp Optical Adhesive Film (part.nr. 4360954, Applied Biosystems)
- nuclease-free water
- cDNA from reverse-transcription reaction
- Taqman Gene Expression assays
- Human ACTB (beta actin) Endogenous Control (part-nr.: 4333762F, Applied Biosystems)
- Taqman Universal PCR Master Mix (part-nr.: 4304437)

Protocol

Generate a plate design: Reactions are performed in duplicates (20 ul total volume per reaction with 2 ul of cDNA template). Therefore 4 ul cDNA for the endogenous control reaction plus 4 ul cDNA for each gene-specific assay is needed. E.g. with one

cDNA reaction which is 20 ul 4 genes can be tested (16ul for gene-specific assays plus 4 ul for endogenous) control. **Since some of cDNA is lost throughout pipetting add 1 or 2 ul of nuclease-free water to your cDNA before using it.**

Put the plate design down in your protocol book before you start pipetting!

Example plate design:

		1 2	3 4	5 6	7 8	9 10	11 12
G1	A	S1	S2	S3	S4	S5	S6
	B	S7	H20	empty	empty	empty	empty
G2	C	S1	S2	S3	S4	S5	S6
	D	S7	H20	empty	empty	empty	empty
G3	E	S1	S2	S3	S4	S5	S6
	F	S7	H20	empty	empty	empty	empty
ACTB	G	S1	S2	S3	S4	S5	S6
	H	S7	H20	empty	empty	empty	empty

S1-S7: samples 1 to 7
G1-G3 gene-specific Taqman assays 1-3
ACBT: ACTB endogenous control

Prepare master-mixes for Taqman assays (gene-specific and beta-actin):

The volume of one reaction is 20 ul – the Taqman assays are 20x.

Pipetting scheme:

	1x	**10 x (for 5 samples) + 10 %**
20x Taqman Assay	1 ul	11 ul
2 x Universal PCR MM	10 ul	110 ul
Water	7 ul	77 ul
Total volume	18 ul	198 ul

Keep the master-mix on ice and pipette 18 ul into the according wells of the PCR plate. Use the same 20 ul tip for all wells corresponding to the same Taqman assay. “Pre-wet” the tip before pipetting the first well: Pipette the master-mix one time up and push the solution out. This makes sure that the surface tension of the pipette tip and the subsequent transferred volume for the first well is the same as for all the other wells.

Mix the cDNA (by flicking the tube) and spin down briefly. Pipette 2 ul of cDNA into the corresponding wells. Use a fresh tip for every well.

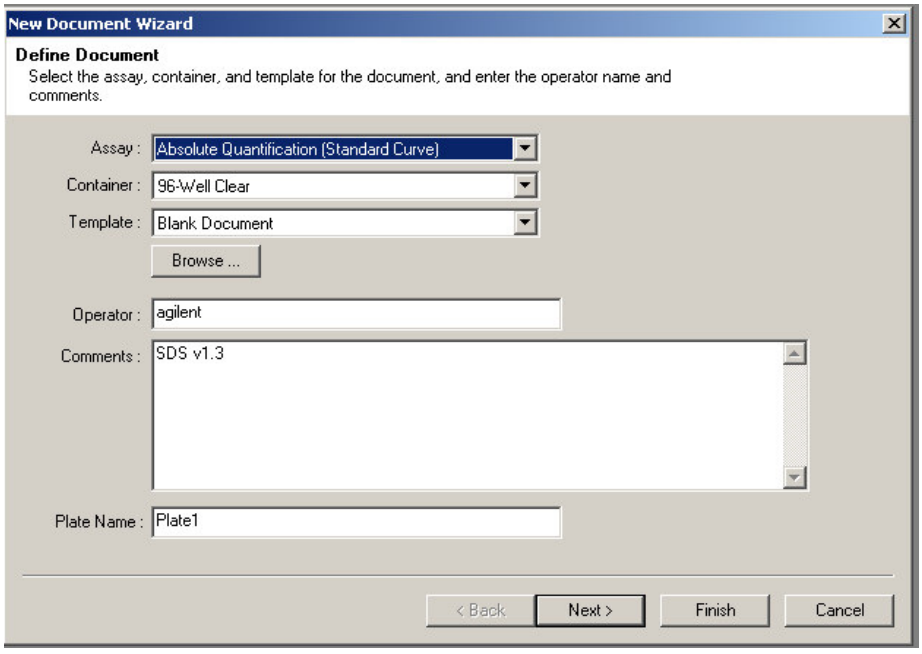
Tip: Use a new box of 0.1-10ul pipetting tips for every new plate. Take out tips from the same position that corresponds to the well on the plate. E.g. take the tip from the top left for well A1 on your plate. Doing this allows to keeping track of the completed wells and prevents mixing up wells.
When finished seal the plate by applying a sheet of Microseal Film. Make sure the film sits firmly on the plate by striking with the plastic tool.

Go quickly to the ABI 7500 cycler in the Microarray Centre. Give the plate two or three short vortex pulses using the plate vortexer next to the plate centrifuges. Make sure the sealing film still sits firmly on the plate. Spin the plate for one minute at 800 rpm to make sure the contents of the wells are at the bottom. Put the plate back on ice.

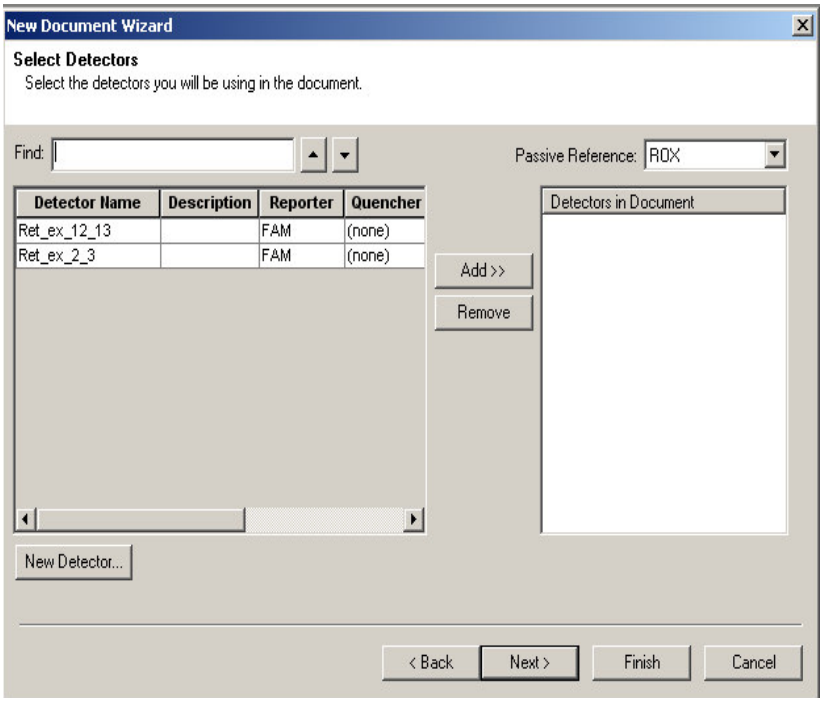
PLEASE NOTE: Replicate consistency is crucial in quantitative RT-PCR which is mostly influenced by pipetting accuracy. Before you use cDNA from tissue samples perform some test runs with cDNA from cell lines in order to make sure your setup works. As a rule of thumb try to do every pipetting step the same way for all wells. Work out a routine which requires some practice!

Set up the PCR machine:

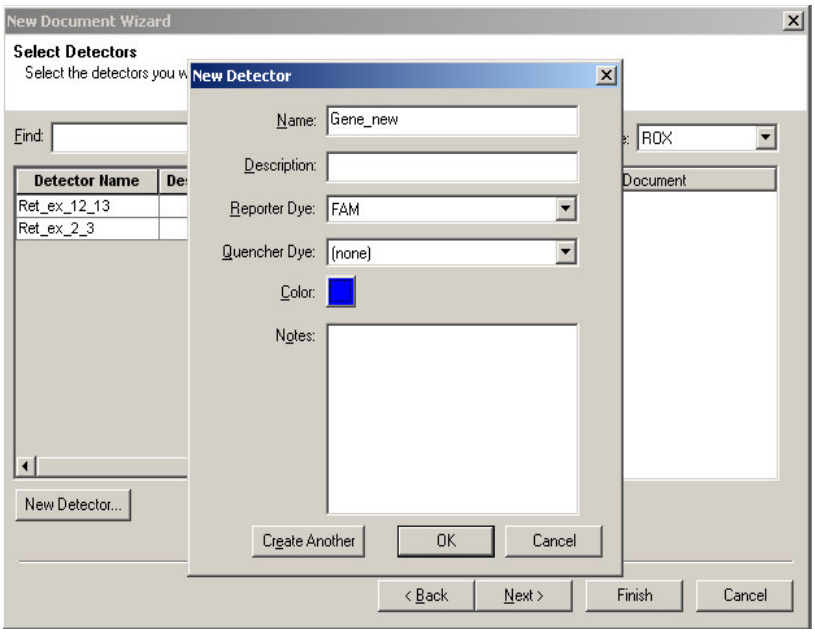
Open the Sequence Detection Software (SDS) from the Desktop of the laptop attached to the PCR machine and click "New Document" at the upper left corner of the programme window. The "New Document Wizard" dialogue opens:



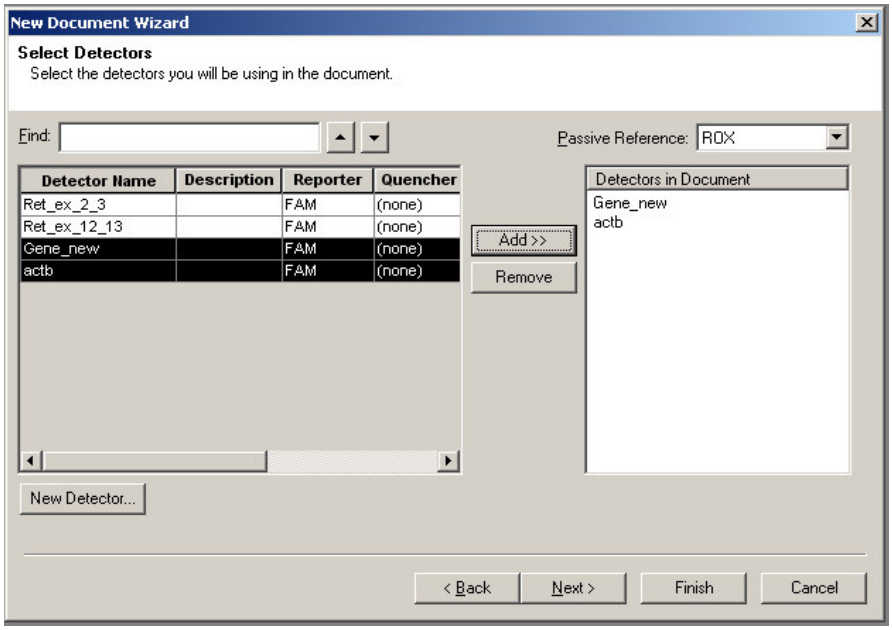
Keep the default selection "Absolute Quantification" and optionally fill in the "Operator", "Comments" and "Plate Name" fields. Click "Next".



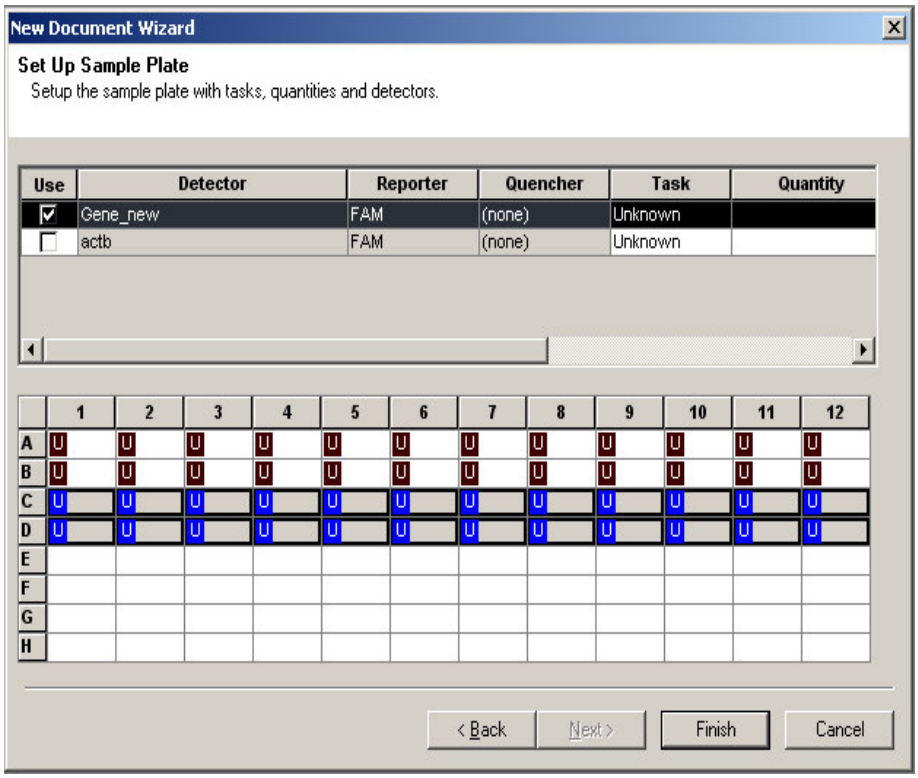
The next window lets you select the Taqman assays you are going to use in combination with your plate. If the assays you are looking for are not in the list click "New Detector..." and create a new Assay in the next dialogue.



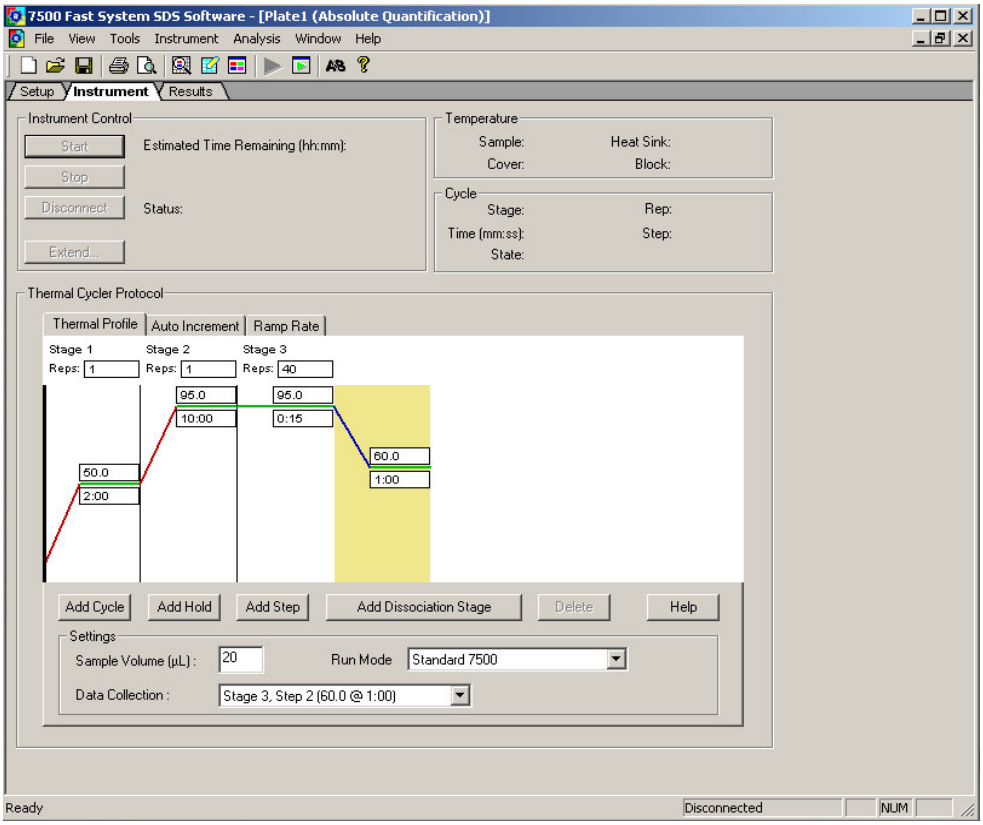
Put in the Name of the assay and optionally a description. Choose a unique colour which makes it easier to interpret curves. Please note that all Taqman assays use FAM as the reporter dye. There is no Quencher dye. Click "OK".



Select the detectors you are going to need and click “Add >>” and “Finish”.



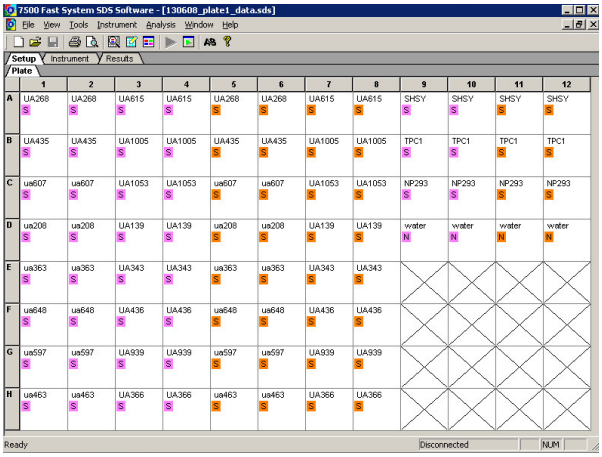
Select Detector and the wells with the according Taqman assay. Click “Finish” and select the “Instrument” tab from the top.



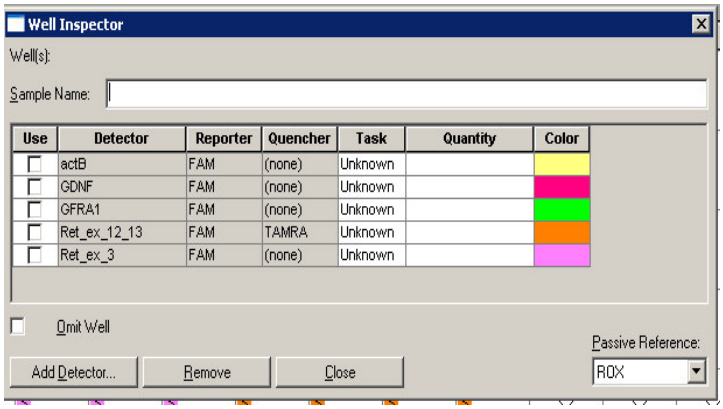
Change the “Run Mode” to “Standard 7500” and save the document. Click “Start”.

The run takes about 1.5 h.

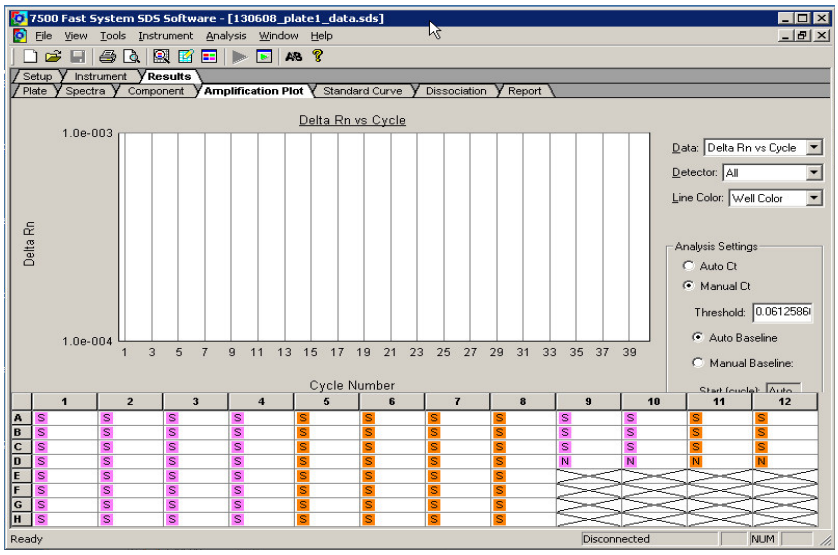
When run has finished go to the “Setup” tab, click the wells and put in the sample names.



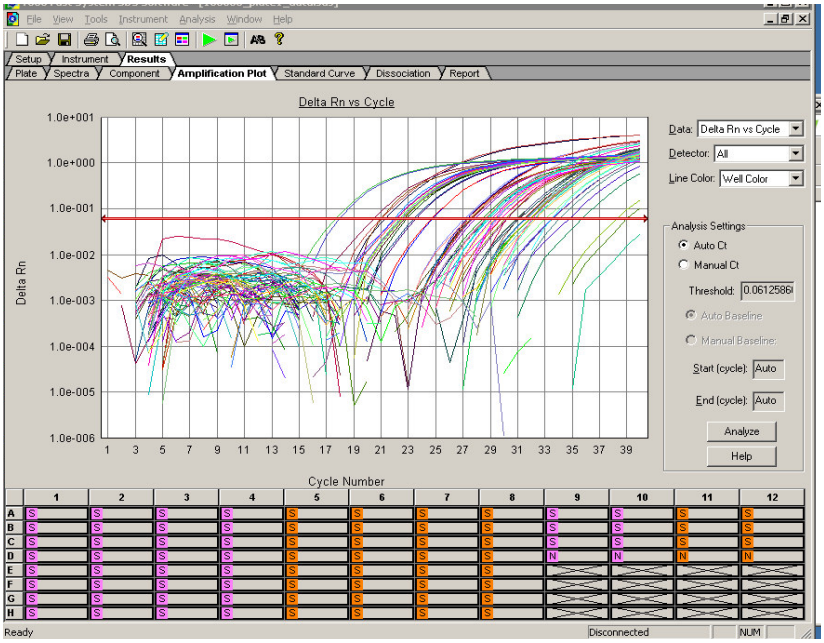
Open the “Well Inspector” by clicking the icon 



Select the wells which are empty and tick the “Omit Well” Box. Then go to the “Results” tab and select the “Amplification Plot” tab.

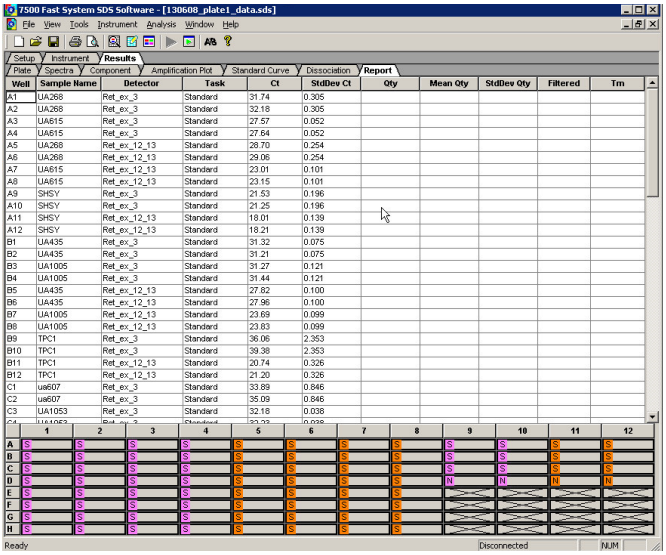


Select all wells and tick “Auto Ct”.



If the programme aligned the red threshold-line properly click “Analyze”. Sometimes the programme has problems finding the threshold. In that case tick “Manual Ct” and adjust the threshold manually somewhere in the linear phase where variation of curves is lowest. Click “Analyze”. Once the data are analysed the colour of the threshold bar turns from red to green.

Now go to the “Report” tab.



In the report tab the results are represented in a table-like format. In order to work with the data you need to export them. You can do that by clicking “File” → “Export” → “Results...”. Browse to the folder you want the results to be saved.

The results are then written into a csv file which can be opened by any spreadsheet application such as Excel or R.