# 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.





#### 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.

#### Author:

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Signature .....

#### Authorised by:

Name Professor GA Thomas
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Signature .....

#### 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.

#### <u>Method</u>

#### 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  $\mu$ l) 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

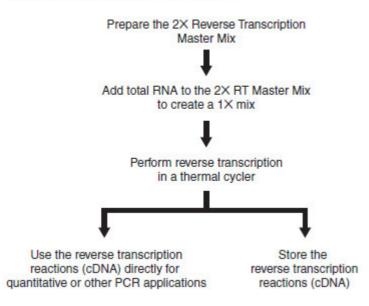
The real-time PCR cycler (ABI 7500) which is based at the MRC Microarray Centre (7<sup>th</sup> floor Commonwealth Building) has to be booked before using it. Put your name and extension number into the book beside the PCR cycler and choose one or more of available time slots. If you cannot use a slot 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**

#### <u>Overview</u>

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



#### <u>Material</u>

- 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

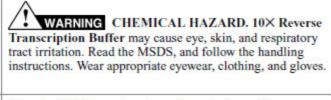
#### <u>Protocol</u>

2.

- 1. label one 0.2 µl tube per sample with sample name and put on the metal plate on ice
  - reactions. Note: Prepare the RT master mix on ice. Volume/Reaction Component (µL) Kit without Kit with **RNase Inhibitor 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 1.0 MultiScribe" 1.0 Reverse Transcriptase RNase Inhibitor 1.0 -Nuclease-free H<sub>2</sub>O 3.2 4.2 Total per Reaction 10.0 10.0

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

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



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

To prepare the cDNA RT reactions:

1.	Pipette 10 $\mu$ L of 2× RT master mix into each well of a 96-well reaction plate or individual tube.
2.	Pipette 10 $\mu$ L 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:

	thermal cyclers list IMPORTANT! The the High Capacity	se conditio	ons are optin	mized for				
		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 vol	lume to 20	μL.					
3.	Load the reactions	into the th	ermal cycle	r.				
-			run.					

#### <u>Storage</u>

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

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

#### Real-Time PCR

#### <u>Material</u>

- 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)

#### <u>Protocol</u>

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		S	2	S	3	S	4	S	5	S	6
GI	В[	S7		H2	20	emp	oty	err	ipty	em	pty	em	pty
G2	C [	S1		S	2	S	3	S	4	S	5	S	6
62	D [	S7		H2	20	emp	oty	err	ipty	em	pty	em	pty
G3	E	S1		S	2	S	3	S	4	S	5	S	6
65	F	S7		H2	20	emp	oty	err	ipty	em	pty	em	pty
ACTB	G	S1		S	2	S	3	S	4	S	5	S	6
AUID	H [	S7		H2	20	emp	oty	err	ipty	em	pty	em	pty

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:

New Document W	Vizard	×
Define Documer Select the assay, comments.	nt , container, and template for the document, and enter the operator name and	
Assay :	Absolute Quantification (Standard Curve)	
Container :	96-Well Clear	
Template :	Blank Document	
	Browse	
Operator :	agilent	
Comments :	SDS v1.3	
	-	
Plate Name :	Plate1	
r late fildine .		
	< Back Next > Finish Cancel	]

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

The second se	ard					×
Select Detectors Select the detectors	you will be using	in the docum	ent.			
Find:			-	Pa	ssive Reference: ROX	•
Detector Name	Description	Reporter	Quencher		Detectors in Document	1
Ret_ex_12_13		FAM	(none)			
Ret_ex_2_3		FAM	(none)	Add >>		
				Remove		
4			Þ			
			Þ			
New Detector	1		Þ			
	]		Þ			
	1		F		t> Finish	

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.

New Document Wizard							×
Select Detectors Select the detectors you w	New Detector				2	<u>&lt;</u>	
Eind: Detector Name Der Ret_ex_12_13 Ret_ex_2_3	<u>N</u> ame: <u>D</u> escription: <u>R</u> eporter Dye: <u>Q</u> uencher Dye: <u>C</u> olor:		3W 		<b>•</b>	8: R0X	•
New Detector	Ngtes: Cr <u>e</u> ate An	other	OK.	<u>N</u> ext	Cancel	Finish	Cancel

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".

New Document Wiza	rd						×
Select Detectors Select the detectors	you will be using	in the docum	ent.				
Eind:			•	Pas	sive Reference:	ROX	•
Detector Name	Description	Reporter	Quencher		Detectors in D	ocument	
Ret_ex_2_3		FAM	(none)		Gene_new		
Ret_ex_12_13		FAM	(none)	Add >>	actb		
Gene_new		FAM	(none)	Auu			
actb		FAM	(none)	Remove			
New Detector	1		Þ				
			< [	<u>}</u> ack <u>N</u> ext	> Fin	ish	Cancel

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

se		Detector			Reporte	r (	Quenche	r	Task	Q	uantity
🗸 Gene	e_new			F.A	٨M	(nor	ne)	Unkr	nown		
actb				FA	M	(nor	ne)	Unkr	nown		
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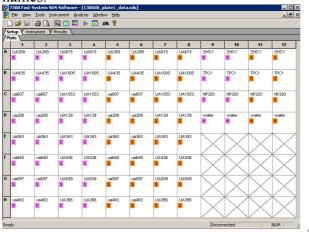
Select Detector and the wells with the according Taqman assay. Click "Finish" and select the "Instrument" tab from the top.

Important       Results         Instrument       Cover:         Start       Extimated Time Remaining (hhrmm):         Start       Start         Start       Start         Start       Start         Start       Start         Start       Start         Start       Start         Block:       Cover:         Block:       Cover:         Block:       Cover:         Start       Start         Extends       Thermal Profile         Auto Increment       Ramp Rate         Stage 1       Stage 2         Stage 3       Stage 3         Respt:       Respt:         Image:	<ul> <li>7500 Fast System SDS Software - [Plate1 (Absolute Quar</li> <li>File View Tools Instrument Analysis Window Help</li> </ul>	ntification)]		_DX _BX
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Settings Sample Volume (µL): 20 Run Mode Standard 7500	10:00 0:16 60.0 1:00			
	Settings			
Data Collection : Stage 3, Step 2 (60.0 @ 1:00)	Sample Volume (µL) : 20 Run Mode	Standard 7500	<b>•</b>	
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Change the "Run Mode" to "Standard 7500" and save the document. Click "Start".

The run takes about 1.5 h.

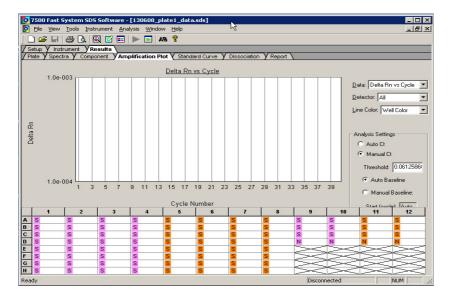
When run has finished go to the "Setup" tab, click the wells and put in the sample names.



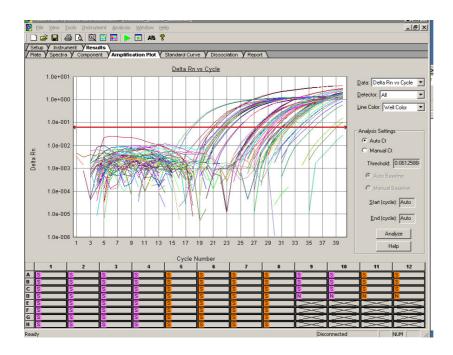
Open the "Well Inspector" by clicking the icon

se	Detector	Reporter	Quencher	Task	Quantity	Color	
	actB	FAM	(none)	Unknown			
	GDNF	FAM	(none)	Unknown			
	GFRA1	FAM	(none)	Unknown			
	Ret_ex_12_13	FAM	TAMRA	Unknown			
	Ret_ex_3	FAM	(none)	Unknown			

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

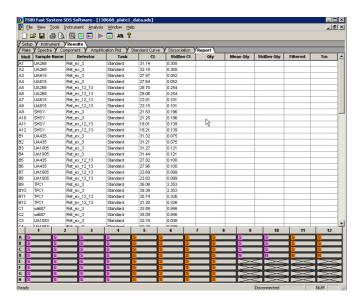


Select all wells and tick "Auto Ct".



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"  $\rightarrow$  "Export"  $\rightarrow$  "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.