AnyGenes®

Efficient technologies for signaling pathways

qPCR SignArrays® 96 Handbook

For all SignArrays® 96 products:

✓ Cat # XXXX**1**-X

(* Cat # XXXX1-X refer to all SignArrays® 96 references developed by AnyGenes®)

For research use only

2019

Summary

I. Introduction

The "qPCR array" technology consists in a high throughput transcriptomic analysis for focused panels of genes, simultaneously in a single step, by real-time quantitative PCR. The SignArrays® system developed by AnyGenes® allows the study of specific signaling pathways of cellular processes (angiogenesis, apoptosis, cell cycle, metabolism of resistance to specific drugs...) or pathological pathways (Alzheimer's disease, multiple sclerosis…). Available on catalog or customized, the SignArrays® 96 system allows to save time and money,thanks to its ease of use in routine, involving shorter delays to get results for research laboratories.

Optimized for a use with the Perfect Master Mix SYBR Green® provided by AnyGenes®, this system based on a very sensitive technology ensures specific, sensitive and reproducible results. The ease of use of SignArrays® system allows routine use, accessible to any research laboratory. All the steps are described in this protocol.

Moreover, AnyGenes® has developed its own analysis tools for results of the SignArrays® system, available on www.anygenes.com. Characterized by its ease of operation,it allows to make a rapid and reliable analysis of your results.

II. Kit contents

SignArrays® 96 products are available in several formats:

Catalog Ref :	Contents
XXXX1-X2S	2 SignArrays® 96 plates with lyophilized primers for focused panels of genes + 2 optical sealing foils
XXXX1-X4S	4 SignArrays® 96 plates with lyophilized primers for focused panels of genes + 4 optical sealing foils
XXXX1-X8S	8 SignArrays® 96 plates with lyophilized primers for focused panels of genes + 8 optical sealing foils
XXXX1-X12S	12 SignArrays® 96 plates with lyophilized primers for focused panels of genes + 12 optical sealing foils
XXXX1-X24S	24 SignArrays® 96 plates with lyophilized primers for focused panels of genes + 24 optical sealing foils

(* Cat # XXXX1 refer to all SignArrays® 96 references developed by AnyGenes®)

We recommend to use the SignArrays® system with our Perfect Master Mix SYBR Green®, optimized together for best specific and reliable results. Moreover, make sure you have ordered the SignArrays® plates compatible with our qPCR instrument before starting the procedure.

Company	Instruments	SignArrays 96® Cat #	Perfect Master Mix SYBR Green® Cat #
Roche	LightCycler® 96	XXXH1-RXS	PMSX-WXS
	LightCycler® 480	XXXH1-RXS	PMSX-WXS
Applied Biosystems	Step One Plus™ Real-Time System	XXXH1-FXS	PMSX-RXS
	ABI 5700	XXXH1-AXS	PMSX-RXS
	ABI 7000	XXXH1-AXS	PMSX-RXS
	ABI 7300	XXXH1-AXS	PMSX-RXS
	ABI 7500 (standard block)	XXXH1-AXS	PMSX-LRXS
	ABI 7500 (FAST block*[2])	XXXH1-FXS	PMSX-LRXS
	ABI 7700	XXXH1-AXS	PMSX-RXS
	ABI 7900 HT (standard block)	XXXH1-AXS	PMSX-RXS
	ABI 7900 HT (FAST block)	XXXH1-FXS	PMSX-RXS
	ViiA7™ system (standard block)	XXXH1-AXS	PMSX-LRXS
	ViiA7™ system (FAST block*[2])	XXXH1-FXS	PMSX-LRXS
	QuantStudio™ 5, 6, 7, 12K Flex System (standard block)	XXXH1-AXS	PMSX-LRXS
	QuantStudio™ 5, 6, 7, 12K Flex System (FAST block)	XXXH1-FXS	PMSX-LRXS
Bio-Rad	iCycler™ iQ	XXXH1-AXS	PMSX-FXS
	iQ™5	XXXH1-AXS	PMSX-FXS
	MyiQ™	XXXH1-AXS	PMSX-FXS
	MyiQ2™	XXXH1-AXS	PMSX-FXS
	Chromo4™	XXXH1-BXS	PMSX-WXS
	Opticon™	XXXH1-BXS	PMSX-WXS
	Opticon2™	XXXH1-BXS	PMSX-WXS
	CFX Connect™	XXXH1-BXS	PMSX-WXS
	CFX96	XXXH1-BXS	PMSX-WXS
	CFX384	-	PMSX-WXS
Eppendorf	Mastercycler™ ep realplex 2	XXXH1-AXS	PMSX-WXS / PMSX-RXS
	Mastercycler™ ep realplex 2S	XXXH1-AXS	PMSX-WXS / PMSX-RXS
	Mastercycler™ ep realplex 4	XXXH1-AXS	PMSX-WXS / PMSX-RXS
	Mastercycler™ ep realplex 4S	XXXH1-AXS	PMSX-WXS / PMSX-RXS
Stratagene / Agilent	Mx3000P™	XXXH1-AXS	PMSX-LRXS
	Mx3005P™	XXXH1-AXS	PMSX-LRXS
	Mx4000™	XXXH1-AXS	PMSX-LRXS
	AriaMx™	XXXH1-AXS	PMSX-LRXS
Techne	Quantica	XXXH1-AXS	PMSX-FXS
	PrimeQ	XXXH1-AXS	PMSX-FXS

* example with Human SignArrays references (XXXH1 for *Homo sapiens*, XXXM1 for *Mus musculus*, XXXR1 for *Rattus norvegicus* species,...)

** LightCycler® 96 and 480 are trademarks of Roche. Step One Plus™ Real-Time System, ABI 5700, ABI 7000, ABI 7300, ABI 7500, ABI 7700,7900HT, ViiA7™ system, QuantStudio™ Systems are trademarks of Applied Biosystems. iCycler™ iQ, iQ™5, MyiQ™, MyiQ2™, Chromo4™, Opticon™, Opticon2™, CFX Connect™, CFX96, CFX384 are trademark of Bio-Rad. Mastercycler™ ep realplex is a trademark of Eppendorf. Mx3000P™, Mx3005P™, Mx4000™ and AriaMx™ are trademarks of Stratagene. Quantica and PrimeQ is a trademark of Techne.

NB: WE INFORM YOU THAT PERFECT MASTER MIX SYBR GREEN® DEVELOPPED BY ANYGENES® (STANDARD HOT-START ENZYMES) ARE NOT SUITABLE FOR USE IN FAST MODE ON APPROPRIATE qPCR INSTRUMENTS, EVEN IF THE SELECTED PLATE FORMAT IS SUITABLE FOR FAST BLOCKS (XXXH1-FXS).

For more product information, please visit **www.anygenes.com** or contact us at **technical@anygenes.com**

III. Storage of AnyGenes® products

SignArrays® 96 plates are lyophilized, which enhances their stability at room temperature. However, for a better and longer storage, it is strongly advised to keep them at -20°C. Therefore, upon receipt, store SignArrays® 96 plates as Perfect Master Mix SYBR Green® kits at -20°C until their use.

These conditions guarantee a long-term storage of AnyGenes® products for a minimum period of six months after their receipt (and 12 months for the SignArrays® 96 plates). Moreover, in order to guarantee the stability of these products, avoid repeated freezing and thawing cycles. If small volumes of Perfect Master Mix SYBR Green® are frequently required, we recommend to stock alicots at -20°C.

IV. Intended use

The SignArrays® 96 system is intended for a research use only and in any case provided for diagnosis, prevention or therapeutic applications. AnyGenes® will be not responsible of the improper use of their products.

V. qPCR SignArrays® 96 plate description

qPCR SignArrays® 96 plates allow transcriptional analysis of 84 specific genes involved in a signaling or pathological pathway in a single qPCR. These plates also include:

- 8 wells for transcriptomic analysis of reference genes, necessary for the normalization step of the results
- and 4 quality controls (positive and negative), allowing validation of the results, obtained for each SignArrays® plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	T01	T02	T03	T04	T05	T06	T07	T08	T09	T10	T11	T12
B	T13	T14	T15	T16	T17	T18	T19	T20	T21	T22	T23	T24
C	T25	T26	T27	T28	T29	T30	T31	T32	T33	T34	T35	T36
D	T37	T38	T39	T40	T41	T42	T43	T44	T45	T46	T47	T48
E	T49	T50	T51	T52	T53	T54	T55	T56	T57	T58	T59	T60
F	T61	T62	T63	T64	T65	T66	T67	T68	T69	T70	T71	T72
G	T73	T74	T75	T76	T77	T78	T79	T80	T81	T82	T83	T84
H	HK1	HK2	HK3	HK4	HK5	HK6	HK7	HK8	QC1	QC2	QC3	QC4

SignArrays® 96 layout

Legend :

T01 atT84 : transcriptomic analysis for the corresponding genes
HK1 : House-keeping genes, reference genes
QC1 à QC4 : quality controls (positive & negative)

Below is an example of SignArray® layout for the human angiogenesis signaling pathway with gene name and sub-pathways. For customized SignArrays®, please contact us.

Angiogenesis SignArrays® Ref : AN1H1

	1	2	3	4	5	6	7	8	9	10	11	12
A	FLT1	KDR	NRP1	NRP2	VEGFA	VEGFC	FIGF	TYMP	PDGFA	PF4	FGF1	FGF2
B	FGFR3	TGFA	TGFB1	TGFB2	TGFBR1	ENG	TNF	IFNA1	IFNB1	IFNG	IL1B	IL6
C	IL8	CCL2	CCL11	CXCL1	CXCL10	CXCL3	CXCL5	CXCL6	CXCL9	TEK	ANGPT1	ANGPT2
D	ANGPTL3	ANGPTL4	EFNA1	EFNA3	EFNB2	EPHB4	ITGAV	ITGB3	CDH5	HAND2	HIF1A	ID1
E	ID3	AKT1	PECAM1	S1PR1	JAG1	TNFAIP2	LEP	LAMA5	ANPEP	PLXDC1	COL18A1	MMP2
F	MMP9	PLAU	PGF	EGF	PROK2	EREG	SPHK1	IGF1	HGF	HPSE	MDK	NOTCH4
G	PTGS2	TIMP1	TIMP2	TIMP3	LECT1	BAI1	STAB1	COL4A3	PLG	SERPINF1	THBS1	THBS2
H	PPIA	ACTB	TBP	B2M	RPLP0	HPRT1	TFRC	GUS	POS CONT	POS CONT	NEG CONT	NEG CONT

- Vascular endothelial growth factors and receptors
- Platelet-derived growth factors and receptors
- Fibroblast growth factors and receptors
- Transforming growth factors and receptors
- Tumor necrosis factor
- Interferons and interleukines
- Chemokines
- Angiopoietines
- Ephrin family members
- Adhesion molecules
- Transcription factors
- Angiogenic factors
- TIMPs
- Negative regulation of angiogenesis
- Reference genes
- Quality Controls

VI. qPCR SignArrays® 96 use protocol

A) Required Reagents and Material

1) Reagents :

- qPCR SignArrays® 96 plates (*supplied with AnyGenes® SignArrays® kit*)
- Perfect Master Mix SYBR Green®
- Ultra-pure & sterile "nuclease, *RNAse, DNAse free*" H_2O (*supplied with AnyGenes® Perfect Master Mix SYBR Green® kit*)

Caution: Do not use DEPC H_2O !!!

- cDNA diluted at the 1/12 from a Reverse Transcription (20µl) performed with 1µg of RNA (for one qPCR SignArrays® 96 plate) or equivalent depending on the used Reverse Transcription kit

2) Material :

- Real-time quantitative PCR instrument (LightCycler® 480 (Roche®), ABI 7900®, ABI 7500® (Applied Biosystems® - Thermo Fisher Scientific®)...) **with 96-well block**
- optical sealing foils compatible with your qPCR instrument (*supplied with AnyGenes® SignArrays® kit*)
- PCR plate centrifuge
- Vortex mixer and Mini-centrifuge
- Pipettes for reaction mix preparation
- Multichannel pipette for dispensing reaction volume of 20µl per well
- "nuclease, RNase, DNase free" tips and tubes
- Disposable reagent reservoirs to collect the reaction mix with multichannel pipettes

B) Important notes

1) Advices before starting protocol

To obtain reliable and reproducible results and avoid contamination and false-positive signals, it is important and necessary to follow Good Laboratory Practices.

- The work area must be thoroughly cleaned before handling to prevent contamination with genomic DNA, whose presence can easily alter the results obtained by quantitative real-time PCR. Decontamination of PCR workstations must regularly be done by exposure to Ultra-Violet (UV) or/and a suitable detergent.

- The pre-PCR, post-PCR and non-PCR workspaces have to be physically separated in the laboratory to avoid contamination by gDNA, cDNA and highly volatile aerosols. It is also preferable to prepare the reaction mix and qPCR SignArrays® 96 plates under workstation.

- The user must wear a lab coat and gloves throughout the procedure and never put his fingers on optical films nor wells of qPCR plates. To avoid this, SignArrays® plates must be handled only by touching the smallest possible areaon the side with gloves.

- Close all your tubes immediately after use and avoid freezing and thawing cycles for all reagents.

Caution : Perfect Master Mix SYBR Green® contains SYBR Green®, a DNA binding dye, which potentially have a carcinogen effect. Therefore, it is strongly essential to avoid inhalation and contact of this product with skin and mucous membranes.

2) Recommendations for cDNA synthesis and preparation

AnyGenes® plates and reagents are subject to strict quality controls to guarantee high-quality and reliable products, allowing a perfect reproducibility of your results.

To perform gene expression studies with the SignArrays® system, it is also important to have high-quality RNA and cDNA. For this, AnyGenes® recommends you to carry out your RNA extraction with Trizol® (Thermo Fisher Scientific®) and Reverse Transcription with the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific®), ideally suited for analysis of SignArrays® plates.

During these steps, it is important to check the quality of your samples. Only an optimal RNA quality ensures reliable and reproducible results obtained by qPCR technology.

Some advices...

- Do not use DEPC H_2O, which could affect the efficiency of the qPCR and previous reactions (especially Reverse Transcription).

- The use of a SignArrays® 96 plate requires almost all the volume of a Reverse Transcription made from **1µg of RNA.** Therefore, for best results, it is necessary to extract RNA from a minimum of $1.10^{\wedge 6}$ cells. Check the approximate yield of RNA from your specific starting material before initiating your experiments. For extraction from tissues, previously assess the equivalency between sample weight and the amount of extracted RNA, which also depends on the studied tissue and the extraction method used : we recommend at least 1 mg of tissue for each experimental condition.

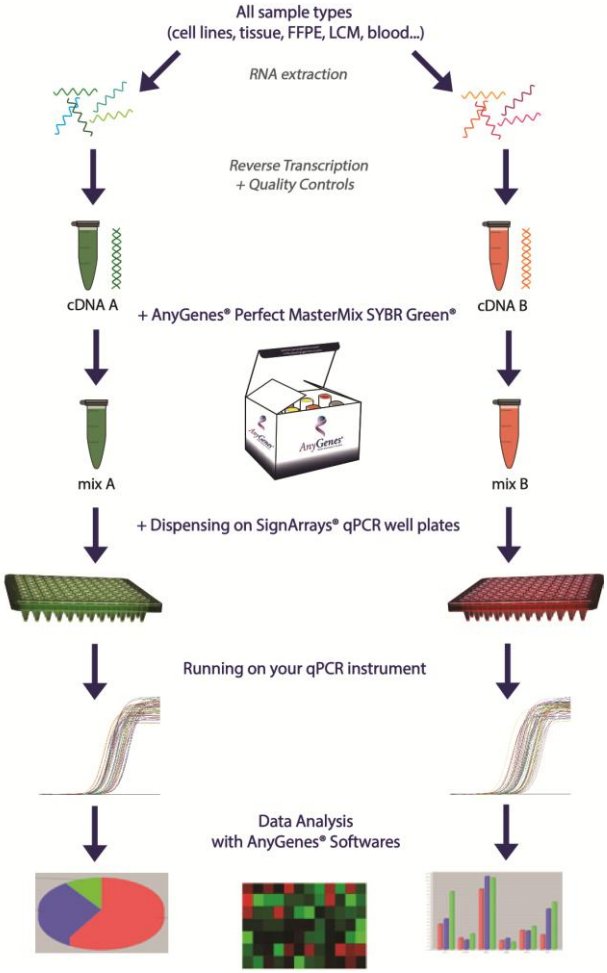
Caution : RNA extraction with a too low amount of cells does not generate good results.

- The extracted RNAs must be quantitatively and qualitatively analysed, especially by checking the quality of A260/A280 absorbance ratios (between 1.8 and 2) and A260/A230 (between 1.7 and 2). The presence of proteins, salts and other contaminants can induce RNA degradation and decrease the qPCR efficiency. That is why the quality of the obtained results with the SignArrays® system directly depends on the quality control of this step.

- Depending on the model of Reverse Transcription from **1µg of RNA** with the High Capacity cDNA Reverse Transcription kit ((Thermo Fisher Scientific®), the final RT reaction volume is 20µl. To perform an **analysis of a sample with a qPCR SignArrays® 96 plate**, cDNA of each Reverse Transcription have to be **diluted at the 1/12 (i.e. a final volume of 240µl)** with ultra-pure H_2O. However, if you use another kit of Reverse Transcription, we recommend to similarly dilute your RT from 1 µg of RNA, to obtain a minimal volume of 240µl, necessary to our SignArrays® system. Please refer you to the manufacturer protocol before starting your reverse transcription.

- The primers design directed by AnyGenes® promotes specific amplification of transcripts of interest and avoids gDNA amplification. However, a treatment step of RNA with DNase, prior to the reverse transcription step, is

recommended in order to ensure the complete absence of gDNA contamination of the samples, which could induce a bias results in the generation of Cq values by the qPCR instrument.



Layout of the transcriptomic analysis procedure with the SignArrays® system

C) Procedure

Before you start...

Caution : In order to obtain reliable and reproducible results, it is essential to respect the same preparation times and protocol for each SignArrays® plate.

- It is also important to prepare the design of your experiments in advance, which allows a better focusing of the user and an optimal handling.

- Make sure the qPCR SignArrays® 96 plate match with the corresponding catalog ref. plate.

1) Thaw AnyGenes® Perfect Master Mix SYBR Green® and your cDNA samples 20 minutes before use, in order that slowly reaches room temperature. However, we strongly advise to work with your samples on ice or cold system.

2) Prepare the work area (highly recommended under workstation) by carefully cleaning all material and areas with a suitable detergent and decontaminating the workstation through exposure to UV.

3) Thaw qPCR SignArrays® 96 plate by checking the catalog ref., carefully remove it of his package and centrifuge for 15-60 s at 1 000 g at room temperature (15-25°C) using a centrifuge, suitable for qPCR plates.

NB : Make sure your SignArrays® plates are compatible with our qPCR instrument before starting the procedure.

4) Meanwhile, briefly centrifuge tubes and reagents and prepare the reaction mix in a 2 ml tube or directly in a disposable reagent reservoir according to the following table:

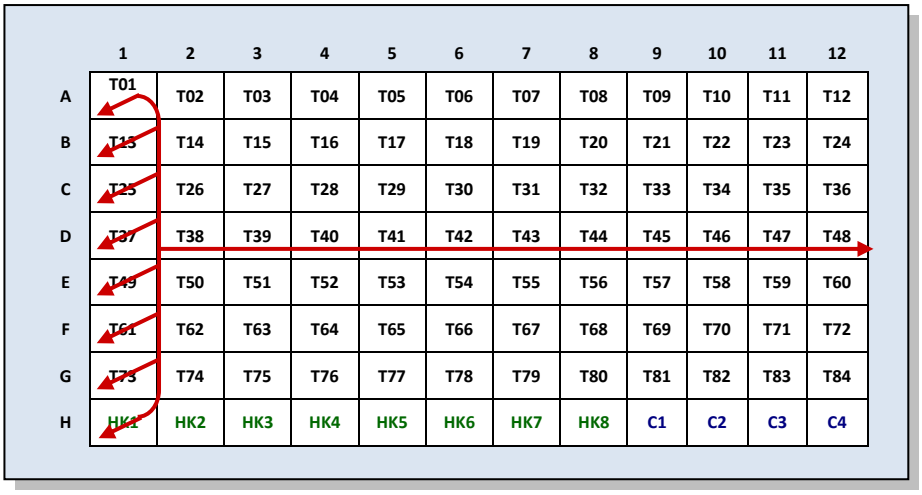
Reagents	**Volumes / reaction**	**Volumes / 1 SignArray® 96** *(= 1 analysed sample)*
2X Perfect Master Mix SYBR Green®	10 µl	**1000 µl**
Ultra-pure H_2O	8 µl	**800 µl**
Diluted cDNA *(at the 1/12)*	2 µl	**200 µl**
Total Volume	**20 µl**	**2000 µl**

**** This protocol can work with 10µl of reaction mix per well. You can adapt the procedure by calculating volumes for each reagent and sample.***

5) Mix thoroughly with a pipette or briefly centrifuge the qPCR mix.

6) Take the qPCR SignArrays® 96 plate out of the centrifuge and remove the adhesive film.

7) Dispense 20µl per well of the reaction mix on the qPCR SignArrays® 96 plate with a multichannel pipette to minimize pipetting errors and, **according to the following format (all wells EXCEPT QC1, QC2, QC3 and QC4)**:



Volume dispensing with an 8 multichannel pipette to avoid cross contaminations

NB : Change tips to avoid cross contamination once it is necessary.

For QC1, QC2, QC3 and QC4 wells (Quality Controls): you have to dispense a qPCR mix **without cDNA** (consisting of 10 µl of 2X Perfect Master Mix SYBR Green® and 10 µl of Ultra-pure H_2O per well).

Reagents	Volumes / reaction	Volumes / 1 SignArray® 96 *(4 QC x 1 analysed sample)*
2X Perfect Master Mix SYBR Green®	10 µl	**50 µl**
Ultra-pure H_2O	10 µl	**50 µl**
Total Volume	**20 µl**	**100 µl**

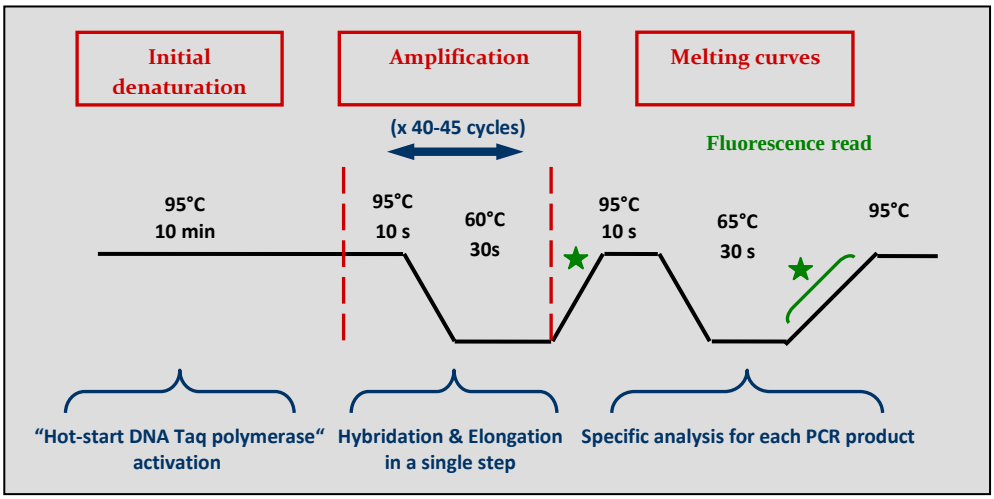
8) Cover the plate with a suitable optical sealing foil (*supplied with the SignArrays® kit*).

9) Centrifuge the SignArray® 15-60 s at 1 000g to remove any bubbles.

10) Meanwhile, prepare and check the run program under the following qPCR conditions (compatible with most of qPCR instruments)*:

Phase	Number of cycles	Time	Temperature	Acquisition mode	Commentaries
Initial denaturation - HOT start Taq activation	**1**	**10 min**	**95°C**	**/**	**"Hot-start DNA Taq polymerase" activation**
Amplification	**40-45**	**10 s**	**95°C**	**/**	**Denaturation of cDNA brands**
		30 s	**60°C**	**quantification**	**Hybridation & elongation steps with fluorescence acquisition**
Melting curves	**1**	**10 s**	**95°C**	**/**	**Melting curves**
		30 s	**65°C**	**/**	
		0 s	**95°C**	**continuous**	

*** Please refer to the manufacturer handbook of your qPCR instrument to adjust the run program.**



Representative scheme of qPCR steps by using qPCR SignArrays® 96 system

11) Withdraw the qPCR SignArrays® 96 plate out of the centrifuge and carefully place it in the qPCR instrument.

Caution : Do not prepare the qPCR SignArrays® 96 plates too early to ensure reliable and reproducible results. However, if your plate was prepared before the start of the qPCR run, keep the plate qPCR SignArrays® 96 on ice or at 4°C in a refrigerator.

12) Start the qPCR run.

D) Data analysis

13) When the run is complete, validate the obtained results thanks to the Cq values (Ct or Cp according to qPCR instruments ; amplification curves) and Tm (melting curves). Then retrieve the raw data.

NB : If you do not use the automatic calculation of Cq values option, please refer to your qPCR instrument manufacturer handbook to define manually the threshold and generate Cq values. Be careful, this step can induce errors if the threshold is not correctly defined.

14) Import these Cq values in our analysis tool based on Excel, free to download in our website. This analysis tool developed by AnyGenes® allows to make a rapid and reliable analysis of your results. Important notes are supplied in the first datasheet to guide you throughout the analysis.

This analysis is based on the "delta delta Cq" (or ΔΔCq) calculation method. Thereby, it allows the comparison between one or more experimental conditions and a reference condition called "control", after having normalized gene expression results with selected reference genes (or House-Keeping Genes).

For more information, please contact us. AnyGenes® team will be pleased to share its experience to help you to generate very high-quality analysis from your SignArrays® data.

- **For positive and negative quality controls:**

The qPCR SignArrays® 384 system contains 2 quality control types for each of the 4 analysed samples :

- **a positive control in duplicates** (QC1 & QC2 wells) : this quality control allows to check 3 parameters :

1. The qPCR efficiency of your experiment : Cq values for QC1 & QC2 wells should be between 26 and 30, according to qPCR instruments and the choice of automatic or manual Cq analysis method.

NB : AnyGenes® don't guarantee these Cq values, so such qPCR efficiency conditions, if you use a qPCR Master Mix not provided by AnyGenes®.

2. Intra-plate repeatability of the qPCR results obtained in the 2 wells (duplicates) : the difference between the 2 Cq values (ΔCq) for QC1 and QC2 wells have to be less than 0.5.

3. Inter-plate repeatability of the qPCR results obtained with several SignArrays® : the difference between the mean of Cq values for QC1 and QC2 wells of each SignArray® have to be less than 1.

In these conditions, the qPCR efficiency is validated, which is the same, regardless analysed samples.

- **a negative control in duplicates** (QC3 & QC4 wells) : Cq values for QC3 & QC4 wells have to be higher than 35 to guarantee the absence of reaction mix contamination.

VII. Additional Information

For any further information, please contact the AnyGenes® technical support via the following email address : technical@anygenes.com

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