Rev.1

GeneQuery™ Human CTLA4 Checkpoint Pathway qPCR Array Kit (GQH-CTL)

Catalog #GK122

Product Description

ScienCell's GeneQuery™ Human CTLA4 Checkpoint Pathway qPCR Array Kit (GQH-CTL) is designed to facilitate gene expression profiling of 88 key genes involved in the human CTLA4 checkpoint pathway and to help identify potential prognostic biomarkers for cancer immunotherapy. CTLA4 is a negative regulator of T cell immune function. It regulates T cell priming in the initial stage of antigen stimulation. CTLA4 can antagonize CD28 signaling in T cells, as CTLA4 and CD28 compete for the same binding ligands, CD80 and CD86. Cancer immunotherapy using CTLA4 immune-checkpoint blockade (ICB) has emerged as a promising strategy to activate antitumor T cell responses, and has led to new immunotherapies for various solid and hematological tumors. Brief examples of how genes may be grouped according to their functions are shown below:

- **CTLA4 downstream immune response:** ZAP70, TRAT1, PTPN11, PIK3R1, PLD1, ARF1, NFKB1, NFATC2, JUN, FOS, BCL2
- **CD28 signaling:** NFATC2, NFKB1/2, GRB2, VAV1, PTPN6, PI3Ks, SNX9, MALT1, CARD11, BCL10, GRAP2
- **AKT signaling / Cell survival:** AKT1/2/3, PTPN2, PTPN6, PTPN11, CDKN1B, BCL2L1, PDPK1, PIK3CA, PIK3CG
- **Co-stimulatory/inhibitory pathway / T cell activation:** CTLA4, CD28, CD80, CD86, PDCD1, CD274, CD276, CD8, CD3, CD4, ICOS, CD27, PDCD1LG2, TNFRSF4/9/18, CSK, LCK, PTCRA
- **Other T cell markers:** BATF, BCL6, CCR6, CD44, CD69, CXCR3/5, HLA-ABC, ICOSLG, IL2RA, IL7R, SELL

***Note*:** all gene names follow their official symbols by the Human Genome Organization Gene Nomenclature Committee (HGNC).

GeneQuery™ qPCR array kits are qPCR ready in a 96-well plate format, with each well containing one primer set that can specifically recognize and efficiently amplify a target gene's cDNA. The carefully designed primers ensure that: (i) the optimal annealing temperature in qPCR analysis is 65°C (with 2 mM Mg^{2+}, and no DMSO); (ii) the primer set recognizes all known transcript variants of target gene, unless otherwise indicated; and (iii) only one gene is amplified. Each primer set has been validated by qPCR with melt curve analysis, and gel electrophoresis.

GeneQuery™ qPCR Array Kit Controls

Each GeneQuery™ plate contains eight controls (Figure 1).

- Five target housekeeping genes (ACTB, GAPDH, LDHA, NONO, and PPIH), which enable normalization of data.

- The Genomic DNA (gDNA) Control (GDC) detects possible gDNA contamination in the cDNA samples. It contains a primer set targeting a non-transcribed region of the genome.
- Positive PCR Control (PPC) tests whether samples contain inhibitors or other factors that may negatively affect gene expression results. The PPC consists of a predispensed synthetic DNA template and a primer set that can amplify it. The sequence of the DNA template is not present in the human genome, and thus tests the efficiency of the polymerase chain reaction itself.
- The No Template Control (NTC) is strongly recommended, and can be used to monitor the DNA contamination introduced during the workflow such as reagents, tips, and the lab bench.

Kit Components

Component	Cat #	Quantity	Storage
GeneQuery™ array plate with lyophilized primers	GK122	1	4°C or -20°C
Optical PCR plate seal	N/A	1	RT
Nuclease-free H_2O	GQ100-1	2	4°C

Additional Materials Required (Materials Not Included in Kit)

Component	Recommended
Reverse transcriptase	First-Strand cDNA Synthesis Master Mix, 4x (ScienCell, Cat #MB6008)
cDNA template	Customers' samples
qPCR master mix	GoldNStart TaqGreen qPCR Master Mix (ScienCell, Cat #MB6018)

Quality Control

All the primer sets are validated by qPCR with melt curve analysis. The PCR products are analyzed by gel electrophoresis. Single band amplification is confirmed for each set of primers.

Product Use

GQH-CTL is for research use only. It is not approved for human or animal use, or for application in clinical or *in vitro* diagnostic procedures.

Shipping and Storage

The product is shipped at ambient temperature. Upon receipt, the plate should be stored at 4°C and is good for up to 12 months. For long-term storage (>1 year), store the plate at -20°C in a manual defrost freezer.

Procedures

Note: The primers in each well are lyophilized.

1. Prior to use, allow plates to warm to room temperature.
2. Briefly centrifuge at 1,500x g for 1 minute before slowly peeling off the seal.
3. Prepare 20 µl PCR reactions for one well as shown in Table 1.

Table 1

cDNA template	**0.2 – 250 ng**
2x qPCR master mix	10 µl
Nuclease-free H_2O	variable
Total volume	***20 µl***

Important: Only *use polymerases with hot-start capability to prevent possible primer-dimer formation.* ***Only*** *use nuclease-free reagents in PCR* amplification.

4. Add the mixture of 2x qPCR master mix, cDNA template, and nuclease-free H_2O to each well containing the lyophilized primers. Seal the plate with the provided optical PCR plate seal.

   Important: In NTC control well, do NOT add cDNA template. Add 2x qPCR master mix and nuclease-free H2O only.

5. Briefly centrifuge the plates at 1,500x g for 1 minute at room temperature. For maximum reliability, replicates are strongly recommended (minimum of 3).

6. For PCR program setup, please refer to the instructions of the master mix of the user's choice. We recommend a typical 3-step qPCR protocol for a 200nt amplicon:

Three-step cycling protocol

Step	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	95°C	10 min	1
Denaturation	95°C	20 sec	40
Annealing	65°C	20 sec	
Extension	72°C	20 sec	
Data acquisition	Plate read		
Recommended	*Melting curve analysis*		1
Hold	4°C	Indefinite	1

7. (Optional) Load the PCR products on 1.5% agarose gel and perform electrophoresis to confirm the single band amplification in each well.

Figure 1. Layout of GeneQuery™ qPCR array kit controls.

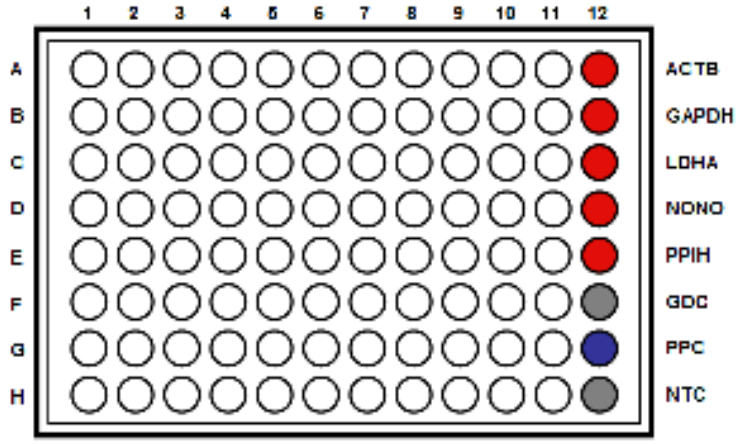


Table 2. Interpretation of control results:

Controls	***Results***	***Interpretation***	***Suggestions***
Housekeeping gene controls	Variability of a housekeeping gene's Cq value	The expression of the housekeeping gene is variable in samples; cycling program is incorrect	Choose a constantly expressed target, or analyze expression levels of multiple housekeeping genes; use correct cycling program and make sure that all cycle parameters have been correctly entered
gDNA Control (GDC)	Cq ≥ 35	No gDNA detected	N/A
	Cq < 35	The sample is contaminated with gDNA	Perform DNase digestion during RNA purification step
Positive PCR Control (PPC)	Cq > 30; or The Cq variations > 2 between qPCR Arrays.	Poor PCR performance; possible PCR inhibitor in reactions; cycling program incorrect	Eliminate inhibitor by purifying samples; use correct cycling program and make sure that all cycle parameters have been correctly entered
No Template Control (NTC)	Positive	DNA contamination in workflow	Eliminate sources of DNA contamination (reagents, plastics, etc.)

Figure 2. A typical amplification curve showing the amplification of a qPCR product.

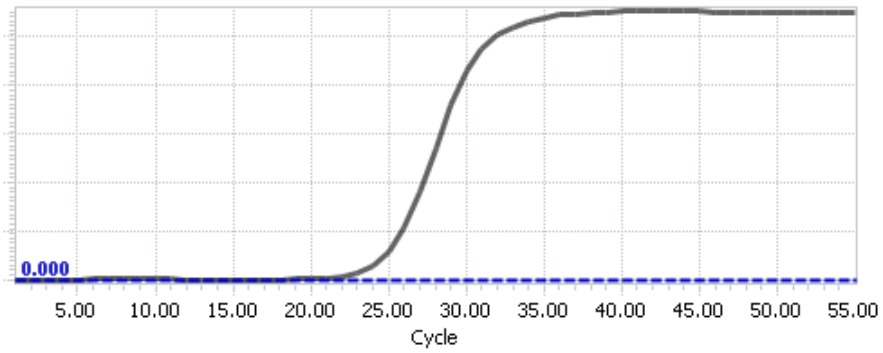
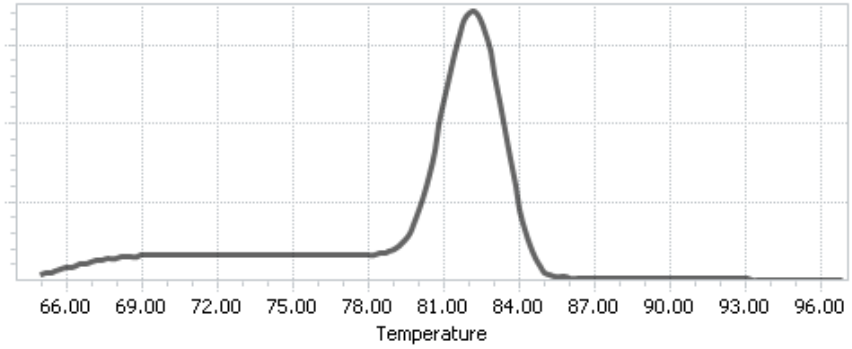


Figure 3. A typical melting peak of a qPCR product.



Quantification Method: Comparative ΔΔCq (Quantification Cycle Value) Method

1. **Note:** Please refer to your qPCR instrument's data analysis software for data analysis. The method provided here serves as guidance for quick manual calculations.

   You can use one or more housekeeping genes as a reference to normalize samples.

   Important: We highly recommend using all 5 housekeeping genes included in this kit: ACTB, GAPDH, LDHA, NONO, and PPIH.

2. For a single housekeeping gene, ΔCq (ref) is the quantification cycle number change for that housekeeping gene (HKG) between an experimental sample and control sample.

   $$\Delta Cq\ (ref) = Cq\ (HKG, experimental\ sample) - Cq\ (HKG, control\ sample)$$

   When using multiple housekeeping genes as a reference, we recommend normalizing using the geometric mean [1] of the expression level change, which is the same as normalizing using the arithmetic mean of ΔCq of the selected housekeeping genes.

   ΔCq (ref) = average (ΔCq (HKG1), ΔCq (HKG2),....., ΔCq (HKG n)) (n is the number of housekeeping genes selected)

   If using all 5 housekeeping genes included in this kit (ACTB, GAPDH, LDHA, NONO, and PPIH) use the following formula:

   $$\Delta Cq\ (ref) = (\Delta Cq(ACTB)+\Delta Cq(GAPDH)+\Delta Cq(LDHA)+\Delta Cq(NONO)+\Delta Cq(PPIH)) /5$$

   Note: ΔCq (HKG) = Cq (HKG, experimental sample) - Cq (HKG, control sample), and ΔCq (HKG) value can be positive, 0, or negative.

3. For any of your genes of interest (GOI),

   $$\Delta Cq\ (GOI) = Cq\ (GOI, experimental\ sample) - Cq\ (GOI, control\ sample)$$

   $$\Delta\Delta Cq = \Delta Cq\ (GOI) - \Delta Cq\ (ref)$$

   $$Normalized\ GOI\ expression\ level\ fold\ change = 2^{-\Delta\Delta Cq}$$

References

[1] Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. (2002) “Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.” *Genome Biol.* 3(7): 1-12.

Example: Comparative ΔΔCq (Quantification Cycle Value) Method

Table 3. Cq (Quantification Cycle) values of 2 genes-of-interest and 5 housekeeping genes obtained for experimental and control samples.

	Genes of Interest		Housekeeping Genes				
Samples	**GOI1**	**GOI2**	***ACTB***	***GAPDH***	***LDHA***	***NONO***	***PPIH***
Experimental	21.61	22.19	17.16	17.84	20.12	19.64	26.40
Control	33.13	26.47	18.20	18.48	20.57	19.50	26.55

$\Delta Cq\ (ref) = (\Delta Cq(ACTB)+\Delta Cq(GAPDH)+\Delta Cq(LDHA)+\Delta Cq(NONO)+\Delta Cq(PPIH)) /5$
$= ((17.16-18.20)+(17.84-18.48)+(20.12-20.57)+(19.64-19.50)+(26.40-26.55))/5$
$= -0.43$

$\Delta Cq\ (GOI1) = 21.61 - 33.13$
$= -11.52$

$\Delta Cq\ (GOI2) = 22.19 - 26.47$
$= -4.28$

$\Delta\Delta Cq\ (GOI1) = \Delta Cq\ (GOI1) - \Delta Cq\ (ref)$
$= -11.52 - (-0.43)$
$= -11.09$

$\Delta\Delta Cq\ (GOI2) = \Delta Cq\ (GOI2) - \Delta Cq\ (ref)$
$= -4.28 - (-0.43)$
$= -3.85$

Normalized GOI1 expression level fold change $= 2^{-\Delta\Delta Cq\ (GOI1)}$
$= 2^{11.09}$
$= 2180$

Normalized GOI2 expression level fold change $= 2^{-\Delta\Delta Cq\ (GOI2)}$
$= 2^{3.85}$
$= 14.4$

Conclusion: Upon treatment, expression level of GOI1 increased 2,180 fold, and expression level of GOI2 increased 14.4 fold.

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GeneQuery™ Human CTLA4 Checkpoint Pathway qPCR Array Plate Layout*
(***8 controls*** in Bold and Italic)

***Note*:** all gene names follow their official symbols by HGNC

	1	2	3	4	5	6	7	8	9	10	11	12
A	AKT1	BCL6	CD28	CD86	EOMES	HLA-B	ICOS	MALT1	PDCD1LG2	PLD1	TBX21	***ACTB***
B	AKT2	CARD11	CD3D	CD8A	FOS	HLA-C	ICOSLG	MKI67	PDPK1	PRDM1	TGFB1	***GAPDH***
C	AKT3	CCR6	CD3E	CD8B	FOXP3	HLA-DPA1	IL2RA	MYC	PIK3CA	PTCRA	TNFRSF18	***LDHA***
D	ARF1	CD160	CD3G	CDKN1B	GATA3	HLA-DPB1	IL7R	NCOR2	PIK3CB	PTPN11	TNFRSF4	***NONO***
E	BATF	CD247	CD4	CSK	GRAP2	HLA-DQA1	JUN	NFATC2	PIK3CD	PTPN2	TNFRSF9	***PPIH***
F	BCL10	CD27	CD44	CTLA4	GRB2	HLA-DQB1	KLRB1	NFKB1	PIK3CG	PTPN6	TRAT1	***GDC***
G	BCL2	CD274	CD69	CXCR3	HAVCR2	HLA-DRA	LAG3	NFKB2	PIK3R1	SELL	VAV1	***PPC***
H	BCL2L1	CD276	CD80	CXCR5	HLA-A	HLA-DRB1	LCK	PDCD1	PIK3R2	SNX9	ZAP70	***NTC***

* gene selection may be updated based on new research and development

Appendix. Plate type choice chart.

Plate type A

Brand	Model	kit catalog #
ABI / Life Tech	ABI 5700	GK122-A
	ABI 7000	GK122-A
	ABI 7300	GK122-A
	ABI 7500	GK122-A
	ABI 7700	GK122-A
	ABI 7900 HT	GK122-A
	QuantStudio	GK122-A
	ViiA 7	GK122-A
Bio-Rad	Chromo4	GK122-A
	iCycler	GK122-A
	iQ5	GK122-A
	MyiQ	GK122-A
	MyiQ2	GK122-A
Eppendorf / Life Tech	Matercycler ep realplex 2	GK122-A
	Matercycler ep realplex 4	GK122-A
Stratagene	MX3000P	GK122-A
	MX3005P	GK122-A

Plate type B

Brand	Model	kit catalog #
ABI / Life Tech	ABI 7500 Fast	GK122-B
	ABI 7900 HT Fast	GK122-B
	QuantStudio Fast	GK122-B
	StepOnePlus	GK122-B
	ViiA 7 Fast	GK122-B
Bio-Rad	CFX Connect	GK122-B
	CFX96	GK122-B
	DNA Engine Opticon 2	GK122-B
Stratagene	MX4000	GK122-B

Plate type C

Brand	Model	kit catalog #
Roche	Lightcycler 96	GK122-C
	Lightcycler 480 (96-well)	GK122-C