NuRNA™ Human Central Metabolism PCR Array

Cat#: AS-NM-004

Instruction Manual Version 1.0

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Table of Contents

I. Introduction

A. Overview

Metabolism impacts all cellular functions and plays a fundamental role in biology. The metabolic pathway architecture varies with cell fates such as growth, proliferation, differentiation[1], and death[2], which is determined by the tissue function and extracellular environment. While diverse mechanisms, for example via signaling pathways, are responsible for the regulation of metabolism, metabolism can reciprocally influence these signals and control cell fate independently[3]. Recent discoveries have given insight of metabolic control of cellular processes such as epigenetics[4, 5], autophagy[6, 7], apoptosis and regulated necrosis[8]. Abnormal metabolism is at the heart of some serious health problems such as obesity, diabetes and cancer. Comprehensive understanding of aberrant metabolic pathways in the diseases will help to find clinical biomarkers and potential therapeutical targets. NuRNA™ Human Central Metabolism PCR Array is specifically designed for rapid, accurate, and systematic expression profiling of 373 enzymes or protein factors in the core metabolic pathways as well as key metabolite transporters, for studying cell metabolism, metabolic regulation, and metabolic changes in biological processes or diseases.

B. Metabolism review

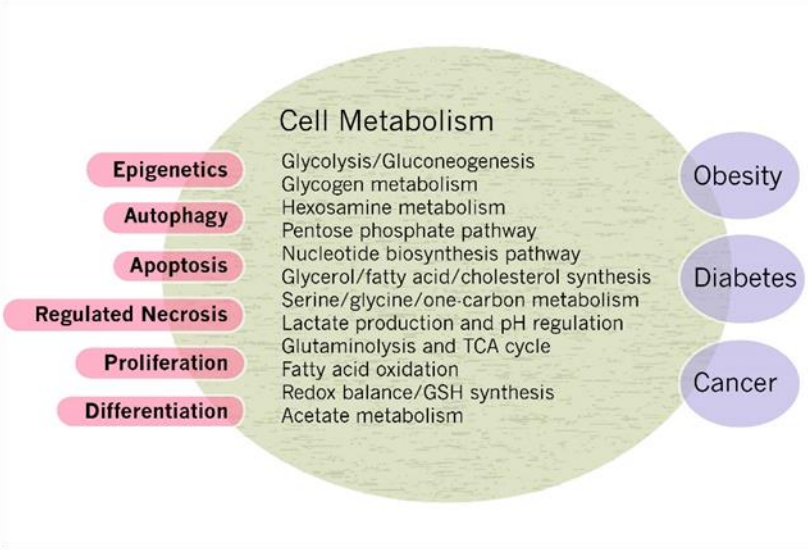


Figure 1 | Metabolic regulation of cellular processes and disease associations.

Metabolic pathways are usually divided into two categories: catabolic and anabolic pathways. Catabolic pathways breakdown macronutrients or storage macromolecules such as glycogen and triglycerides, and ultimately utilize the fuels via glycolysis, tricarboxylic acid cycle (TCA) and β oxidation. Anabolic pathways build upon basic monomers (sugar, fatty acids and amino acids) into macromolecules (polysaccharides, lipids and proteins) that support cellular growth, proliferation, tissue regeneration, and energy storage. Cells adapt

the metabolism to different physiological states and maintain homeostasis by adjusting transitions between anabolic and catabolic processes.

The metabolic requirements for basic metabolic currencies (*e.g.* ATP, acetyl-CoA, NADH, NADPH) are determined by the cell type, tissue function and extracellular environment. For example, when quiescent immune cells are stimulated for rapidly cell proliferation, the cells shift from low to high nutrient uptake with activation of anabolic pathways. On the other hand, differentiated cardiac myocytes do not proliferate but rely heavily on oxidative phosphorylation to meet the high demand for ATP. Different from the above cell types, hepatocytes perform chores of metabolic processes to synthesize glucose, amino acids, and macromolecules while also recycle metabolic byproducts from other tissues and convert waste products for excretion. Each of these tissue physiologies requires different compositions of metabolic currencies and runs unique regulatory circuits.

Nutrient availability to the cells can also heavily impact the metabolism. For instance, hepatocytes within a gradient of oxygen and nutrients imparted by the portal architecture of the liver have different utilization of glucose oxidation vs amino acids or fatty acids to fuel mitochondrial respiration. When other nutrients are limited, consumption of macromolecules as a source of fuel during macroautophagy will activate catabolic pathways to maintain energy homeostasis.

Intermediate metabolism and signal transduction are deeply intertwined at several levels. Many proteins that mediate vital metabolic functions also have key activities in the transduction of cell regulatory signals. Metabolic checkpoints are equipped to sense metabolic variables and generate a biological response through transducers and effectors. Such basic checkpoints are highly interconnected, making up a complex network that controls cell fate in response to metabolic perturbations. Many metabolic pathways play critical regulatory roles in biological processes such as epigenetics, autophagy and cell death (Fig. 1).

■ Metabolic control of epigenetics

In addition to primary DNA sequence, much of the information regarding when and where to initiate transcription is stored in covalent modifications of DNA and its associated proteins. The patterns of various chromatin modifications, such as DNA cytosine methylation and hydroxymethylation, histone acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation at the lysine (K) or arginine (R) residues, determine the genomic active states. The complexity and dynamics of epigenetic modifications provide a link between the extracellular environment and nuclear transcription. Most chromatin-modifying enzymes require substrates or cofactors that are intermediates of cell metabolism, whose availability and concentrations could modulate the activities of chromatin-modifying enzymes and therefore influence chromatin dynamics. Such metabolites, and often the enzymes that produce them, can transfer into the nucleus, directly linking metabolism to nuclear transcription. For example, Acetyl-CoA is the sole donor of acetyl groups for acetylation in eukaryotic cells. Acetyl-CoA is synthesized in the cytoplasm and nucleus from acetate, citrate or pyruvate by acyl-CoA synthetase short-chain family member 2 (ACSS2), ATP-citrate lyase (ACLY) and pyruvate dehydrogenase complex (PDC). Because Acetyl-CoA is very unstable and cannot easily cross cellular membranes, acetylation is compartmentalized and must occur very close to the site where acetyl-CoA is produced. ACLY and ACSS2 are present in the nucleus in addition to the cytoplasm, having the ability regulate overall histone acetylation levels[9, 10]. Additionally, PDC can dynamically translocate from mitochondria to nuclei following serum, EGF stimulation or mitochondrial stress, where it produces acetyl-CoA to promote histone acetylation[11]. These examples illustrate metabolite-producing enzymes and their participating metabolic pathways can be important regulators of epigenetics.

■ Metabolic control of autophagy

The primary, phylogenetically conserved role of autophagy is to maintain cellular homeostasis in conditions of dwindling nutrient supplies and other metabolic perturbations (e.g., hypoxia). This is achieved through the rapid mobilization of endogenous reserves, aimed at retrieving fuel for ATP synthesis as well as building blocks for essential anabolic reactions. The process is coupled to a global rewiring of intracellular metabolism. Autophagy is triggered by changes in the availability of nutrients in extracellular fluids, including drops in the levels of glucose, amino acids[12], acetyl-CoA[13], iron[14] and the relative abundance of reduced versus oxidized NAD[15], or the accumulation of specific lipids[16, 17] and ammonia as the product of amino acid catabolism[18]. Many of these metabolic cues stimulate autophagy by their inhibition of the rapamycin complex 1 (mTORC1) targets; inhibition of various acetyltransferases; or activation of AMP-activated protein kinase (AMPK), deacetylases of the sirtuin family, or eukaryotic translation initiation factor 2a (eIF2a) kinases. In addition, the accumulation of lipids may directly favor the formation of autophagosomes in a patatin-like phospholipase-domain containing 5 (PNPLA5)-dependent fashion[16]. The depletion of iron has autophagy-stimulating effects upon the recognition of ferritin heavy and light chains by the autophagic adaptor nuclear receptor coactivator 4 (NCOA4) [14]. Both mTORC1 and AMPK regulate autophagy by controlling the activity of essential components of the autophagic machinery, such as unc-51 like autophagy activating kinase 1 (ULK1)[19] or Beclin 1 (BECN1)[20]. Moreover, they are both involved in mutually regulatory interactions as well as in functional interactions with other nutrient sensors, such as sirtuins.

■ Metabolic control of cell death

When metabolic perturbations are excessively severe or protracted in time, metabolic checkpoints become capable of initiating apoptotic or necrotic processes of cell death. Major cellular organelles with metabolic functions, particularly mitochondria, can determine whether the cell response to the stress to be adaptive or suicidal. Mitochondria continuously adapt their shape to the changing bioenergetics demands as they are subjected to quality control by autophagy, or they can undergo a lethal permeabilization process that initiates apoptosis. Other metabolic checkpoints include AMPK-TORC1 checkpoint, autophagy checkpoint, acetyl-CoA/CoA checkpoint, HIF-1 checkpoint, ER stress checkpoint, p53 and nonessential amino acid checkpoint, lipid synthesis checkpoint, PARP checkpoint and metabolic enzymes checkpoint (such as PKM2 and GAPDH)[2]. Metabolic checkpoints are in place to monitor metabolic signals indicating the nutrient availability or the state of the intracellular metabolic pathways. Key metabolic signals include ATP/ADP, acetyl-CoA/CoA, NAD+/NADH and NADP+/NADPH ratios, as well as the amounts of lipid products, glycosylated proteins, and reactive oxygen species (ROS). Multiple proteins in the metabolic circuitries, including oxidative phosphorylation and metabolite transport across membranes, may participate in the regulated or catastrophic dismantling of organelles. For example, holocytochrome c required for oxidative phosphorylation and mitochondrial ATP synthesis also promotes the lethal activation of caspases in response to mitochondrial outer membrane permeabilization[2].

■ Metabolism and Cancer

The characteristic metabolism in cancer cells is one of the hallmarks of cancer[21]. The profoundly altered metabolism, mainly driven by oncogenic signaling pathways or metabolic enzymes amplified or alternatively spliced in cancer, allows cancer cells to meet the metabolic demand of sustained growth, proliferation, and survival in nutrient fluctuating environment[22]. Enhanced uptake of glucose, a common feature of cancers, supports the production of intermediates for the synthesis of lipids, proteins and nucleic acids. The increased glutamine uptake and glutaminolysis replenish the intermediates in the TCA cycle that are redirected to biosynthetic reactions. The increased biosynthetic activity of cancer cells also requires a corresponding increase in the supply of NADPH as a reducing agent for anabolic reactions and to maintain cellular redox balance [23]. The tumorigenic transformation and cancer progression involve epigenetic alterations, which are dependent on the metabolites such as acetyl-CoA for acetylation, NAD for deacetylation, SAM for methylation,

α-ketoglutarate for demethylation, and UDP-GlcNAc for glycosylation, as discussed in previously in the "Metabolic control of epigenetics" Section.

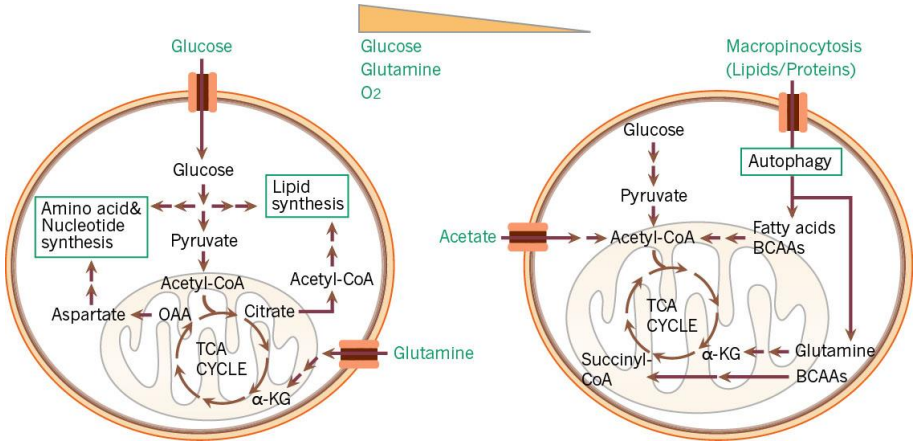


Figure 2 | Cancer metabolism shifts from nutrient and oxygen access (left) to deprivation (right).

Within solid tumors, regional metabolic activities vary significantly with the microenvironment, particularly the access to nutrients and oxygen[22] (Fig. 2). Cells located close to vasculature have access to nutrients (such as glucose and glutamine) and oxygen to feed oncogene-stimulated anabolic pathways. Enhanced uptake of glucose supports the production of intermediates for the synthesis of lipids, proteins and nucleic acids, while the increased glutamine and glutaminolysis replenish intermediates of TCA cycle that are redirected into biosynthetic reactions. These macromolecular biosyntheses allow the biomass accumulation during tumor growth and proliferation. However, at sites where nutrient and oxygen availability dwindles within the tumor mass, the metabolism shifts to alternative pathways for macromolecular degradation to support cell survival. Cancer cells accumulate tumorigenic mutations that stimulate autophagy and micropinocytosis, which facilitate lipid scavenging and fatty acid oxidation to sustain their viability. Acetate and branched amino acids (BCAA) are also used as alternative metabolic substrates under hypoxic and nutrient deficiency conditions.

C. Product summary

■ Array contents

NuRNA™ Human Central Metabolism PCR Panel contains qPCR assays to profile the transcripts expression of 373 genes for the enzyme and protein in the core metabolic pathways, including crucial metabolite transporters. These genes are comprehensively compiled from scientific publications and multiple metabolic pathway databases. Five Housekeeping genes for qPCR normalization, one RNA Spike-In for overall efficiency, three Positive PCR Control (PPC) for PCR efficiency and inter-plate calibration, and Genomic DNA Control (GDC) for genomic DNA contamination detection are included as the controls.

One 384-well PCR panel contains one array for one sample in a 384-well (1*384) format.

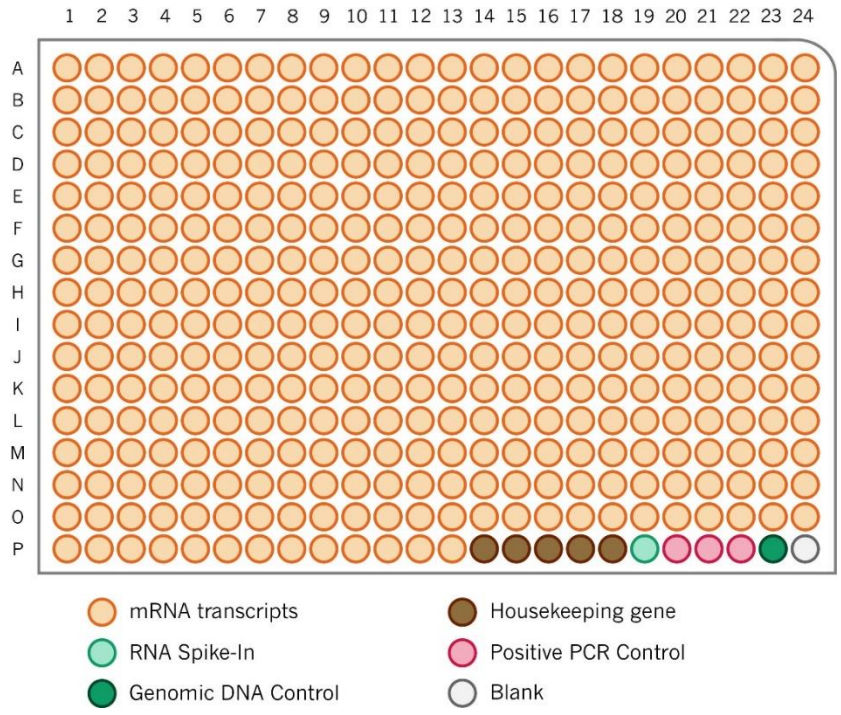


Figure 3 | The 384-well plate layout for NuRNA™ Human Central Metabolism PCR Array

Table 1 | List of human central metabolism transcripts and the Controls

1	SLC2A1	65	SLC16A10	129	SLC27A2	193	PDHA1	257	ACADM	321	TK1
2	SLC2A2	66	SLC16A11	130	SCD	194	PDHA2	258	ACADL	322	TK2
3	SLC2A3	67	SLC16A12	131	SCD5	195	PDHB	259	ACADSB	323	TYMP
4	SLC2A4	68	G6PC	132	FADS1	196	DLAT	260	ACAD8	324	TYMS
5	SLC2A5	69	G6PC2	133	FADS2	197	PDHX	261	ACAD9	325	DTYMK
6	SLC2A6	70	G6PC3	134	ACAT1	198	PDK1	262	ACAD10	326	PPAT
7	SLC2A7	71	FBP1	135	ACAT2	199	PDK2	263	ACAD11	327	GART
8	SLC2A8	72	FBP2	136	HMGCR	200	PDK3	264	ECHS1	328	PFAS
9	SLC2A9	73	PCK1	137	HMGCS1	201	PDK4	265	EHHADH	329	PAICS
10	SLC2A10	74	PCK2	138	HMGCS2	202	PDP1	266	HADHA	330	ADSL
11	SLC2A11	75	PC	139	PHGDH	203	PDPR	267	HADHB	331	ATIC
12	SLC2A12	76	LDHD	140	PSAT1	204	PDP2	268	HADH	332	ADSSL1
13	SLC2A14	77	GPT	141	PSPH	205	L2HGDH	269	HSD17B10	333	ADSS
14	HK1	78	GPT2	142	GLDC	206	D2HGDH	270	HSD17B4	334	AMPD1
15	HK2	79	BCAT1	143	AMT	207	SLC1A4	271	ACAA1	335	AMPD2
16	HK3	80	BCAT2	144	GCSH	208	SLC1A5	272	ACAA2	336	AMPD3
17	GCK	81	PGM1	145	DHFR	209	SLC38A1	273	ECH1	337	AK2
18	ADPGK	82	PGM2	146	DHFRL1	210	SLC38A3	274	ECI1	338	AK3
19	HKDC1	83	UGP2	147	SHMT1	211	SLC38A5	275	ECI2	339	AK6
20	GPI	84	GYS1	148	SHMT2	212	SLC38A7	276	ETFA	340	PDE4D
21	PFKL	85	GYS2	149	MTHFD1L	213	GLS-isoform 1	277	ETFB	341	ADCY1
22	PFKM	86	GBE1	150	MTHFD1	214	GLS-isoform 2	278	SCP2	342	ADCY2
23	PFKP	87	GFPT1	151	MTHFD2	215	GLS-isoform 3	279	PAFAH1B1	343	ADCY3
24	ALDOA	88	GFPT2	152	MTHFD2L	216	GLS2	280	PAFAH1B2	344	ADCY4
25	ALDOB	89	GNPNAT1	153	MTHFR	217	SLC1A1	281	PAFAH1B3	345	ADCY5
26	ALDOC	90	UAP1	154	MTR	218	SLC1A2	282	ALDH1B1	346	ADCY6
27	TPI1	91	UAP1L1	155	BHMT	219	SLC1A3	283	ALDH2	347	ADCY7
28	GAPDH	92	PGM3	156	MAT1A	220	SLC1A6	284	ALDH3A2	348	ADCY9
29	GAPDHS	93	G6PD	157	MAT2A	221	GOT1	285	ALDH7A1	349	ADCY10
30	PGK1	94	PGD	158	MAT2B	222	GOT2	286	ALDH9A1	350	APRT
31	PGK2	95	PGLS	159	DNMT1	223	GLUD1	287	ACSS1	351	DCK
32	PGAM1	96	H6PD	160	DNMT3A	224	GLUD2	288	ACSS2	352	PNP
33	PGAM2	97	RPIA	161	DNMT3L	225	CBS	289	ACSS3	353	ADA
34	PGAM4	98	RPE	162	DNMT3B	226	CTH	290	ACOT12	354	CECR1
35	BPGM	99	RPEL1	163	AHCYL1	227	GCLC	291	CAD	355	HPRT1
36	ENO1	100	TKT	164	AHCY	228	GCLM	292	DHODH	356	IMPDH1
37	ENO2	101	TKTL1	165	AHCYL2	229	GSS	293	UMPS	357	IMPDH2
38	ENO3	102	TKTL2	166	ACO1	230	GSR	294	CMPK1	358	GMPS
39	PKR	103	TALDO1	167	ACO2	231	SLC7A11	295	CMPK2	359	GUK1
40	PKL	104	PRPS1	168	IDH1	232	NNT	296	CANT1	360	PDE10A
41	PKM2	105	PRPS1L1	169	IDH2	233	ME1	297	RRM1	361	GUCY1A2
42	PKM1	106	PRPS2	170	IDH3A	234	ME2	298	RRM2	362	GUCY1A3
43	PFKFB1	107	RBKS	171	IDH3B	235	ME3	299	RRM2B	363	GUCY1B3
44	PFKFB2	108	GPD1	172	IDH3G	236	CEL	300	NME1	364	GUCD1
45	PFKFB3-isoform 1	109	GPD1L	173	OGDH	237	PNLIPRP3	301	NME2	365	GUCY2C
46	PFKFB3-isoform 2	110	SLC25A1	174	DHTKD1	238	AADAC	302	NME3	366	GUCY2D
47	PFKFB3-isoform 3	111	ACLY	175	OGDHL	239	LIPC	303	NME4	367	GUCY2F
48	PFKFB3-isoform 4	112	ACACA	176	DLST	240	PNLIP	304	NME6	368	GUCA1A
49	PFKFB4	113	ACACB	177	DLD	241	PNLIPRP1	305	NME7	369	GUCA1B
50	LDHA	114	MLYCD	178	SUCLG1	242	PNLIPRP2	306	AK1	370	GUCA1C
51	LDHB	115	FASN	179	SUCLG2	243	PNPLA2	307	AK4	371	GUCA2A
52	LDHC	116	ACSBG1	180	SUCLA2	244	PNPLA3	308	AK5	372	GUCA2B
53	LDHAL6A	117	ACSBG2	181	SDHA	245	LIPF	309	AK7	373	XDH
54	LDHAL6B	118	ACSL1	182	SDHB	246	LIPG	310	AK8	374	ACTB
55	UEVLD	119	ACSL3	183	SDHC	247	LIPE	311	AK9	375	B2M
56	SLC16A1	120	ACSL4	184	SDHD	248	MGLL	312	DUT	376	Gusb
57	SLC16A2	121	ACSL5	185	SDHAF1	249	ABHD12	313	UCKL1	377	Hsp90ab1
58	SLC16A3	122	ACSL6	186	SDHAF2	250	ABHD6	314	NT5C2	378	18S RNA
59	SLC16A4	123	ACSM1	187	SDHAF3	251	CPT1A	315	UPP1	379	RNA Spike-in
60	SLC16A5	124	ACSM2A	188	SDHAF4	252	CPT1B	316	UPP2	380	PPC
61	SLC16A6	125	ACSM2B	189	FH	253	CPT1C	317	GDA	381	PPC
62	SLC16A7	126	ACSM3	190	MDH1	254	CPT2	318	CTPS1	382	PPC
63	SLC16A8	127	ACSM4	191	MDH2	255	ACADVL	319	DCTD	383	GDC
64	SLC16A9	128	ACSM5	192	MDH1B	256	ACADS	320	CDA	384	BLANK CONTROL

Description of the control assays

NuRNA™ Human Central Metabolism PCR Array includes a series of external and internal controls for data quality and accuracy. These controls are described below.

- **HK** (Housekeeping gene internal references): 5 human housekeeping genes ACTB, B2M, Gusb, Hsp90ab1, and 18S rRNA are included as the internal qPCR normalization references. Arraystar PCR system provides multiple reference genes selected among commonly used reference genes by using a stringent bioinformatic algorithm, which offers the flexibility of choosing the most valid reference gene(s) for qPCR normalization for your sample types.

- **RNA Spike-in** (External Control): One External RNA Spike-in Mix is added in the RNA sample prior to the first strand cDNA synthesis. The RNA Spike-in control assay indicates the overall success and the efficiency of the reaction beginning from the cDNA synthesis to the final qPCR. Any problem(s) in these steps will result in a failed or compromised RNA Spike-in outcome. RNA spike-in assay results for samples are compared and outliers or failed reactions may be identified and excluded from further data analysis.

- **PPC** (Positive PCR control)**:** 3 replicates of an artificial DNA and the PCR primer pair to indicate the qPCR amplification efficiency. A Ct value greater than 25 is an indication of low qPCR amplification efficiency. More importantly, the PPC are used as inter-plate calibrator (IPC) between PCR plate runs to give the same Ct value for the calibrator, thereby reducing run-to-run variance. Inter-plate calibration (IPC) can easily be performed with the data analysis tool avaliable on our website (www.arraystar.com).

- **GDC** (Genomic DNA Control): PCR assay for an untranscribed genomic region. Non-RT sample or RNA sample are added during the qPCR Process. The Ct values should be greater than 35. A positive GDC signal indicates the array result may have been compromised with genomic DNA contamination.

Shipping and Storage

Arraystar PCR Arrays are shipped at ambient temperature, on ice, or on dry ice, depending on the destination and accompanying products. Store at –20°C upon receipt. The contents are stable for at least 6 months.

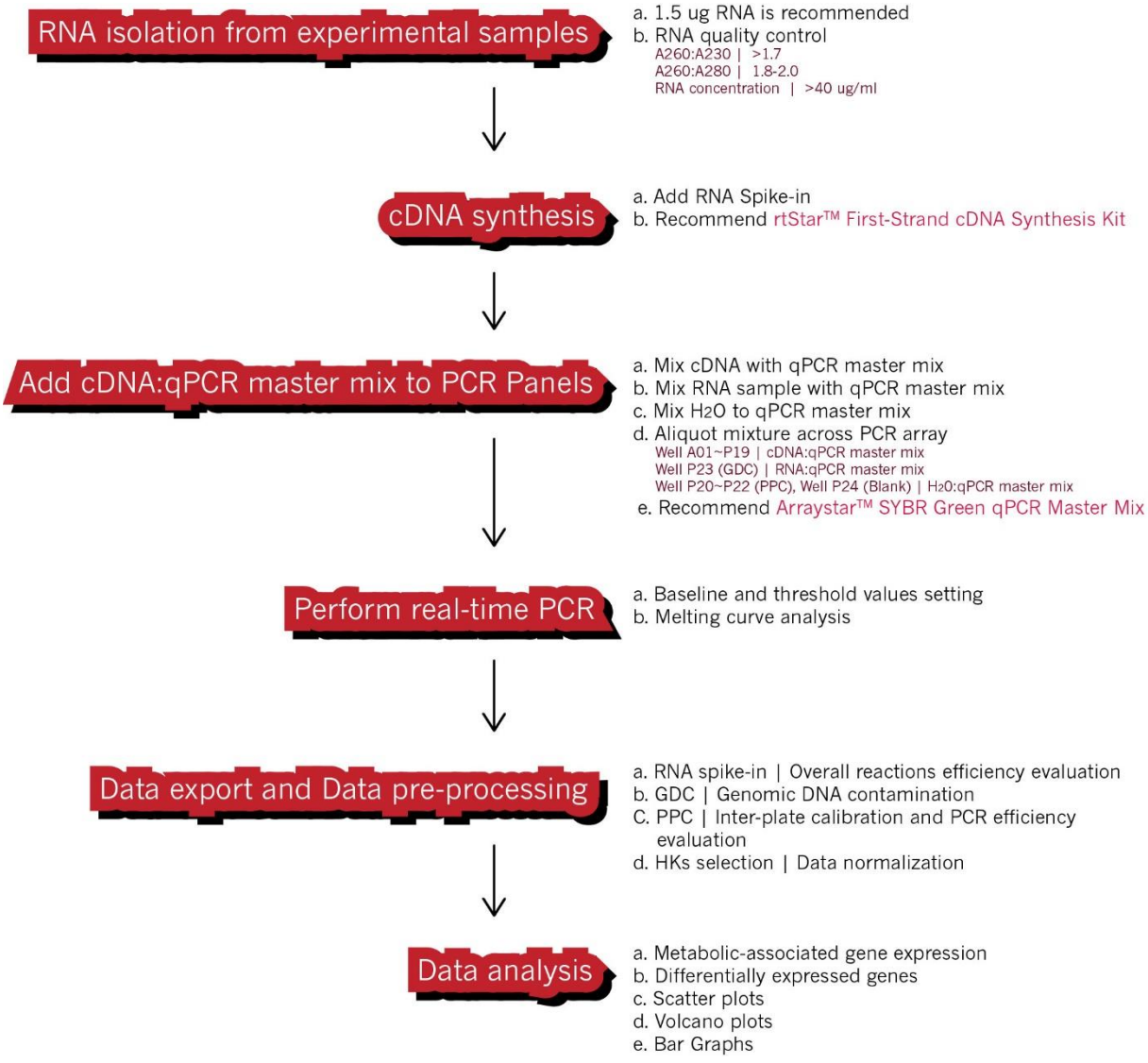
Additional Required Equipment

- Thermal cycler
- Real time qPCR instrument, compatible with 384-well plate format

Additional Required Reagents

- rtStar™ First-Strand cDNA Synthesis Kit (Cat# AS-FS-001)
- Arraystar™ SYBR Green qPCR Master Mix (Cat# AS-MR-006-5)
- Nuclease free PCR-grade water

D. Protocol overview



II. Protocol

IMPORTANT: Please read through this entire protocol before your first experiment. Prepare a workspace and use labwares free of DNA, RNA, and nuclease contamination. Wear gloves while performing this protocol.

A. RNA sample preparation and quality control

For best results, RNA samples must meet the QC standards of integrity and purity from protein, organics, genomic DNA contamination and excessive RNA degradation. You can measure the RNA concentration and purity by UV spectrophotometry. You can also check 18S and 28S ribosomal RNA as an indicator of RNA integrity by denaturing gel electrophoresis or by using an Agilent Bioanalyzer.

- A260:A230 ratio greater than 1.7
- A260:A280 ratio between 1.8 and 2.0
- Total RNA concentration greater than 40 ng/μL

The recommended input total RNA amount is 1.5 μg. Lower amounts may reduce the assay sensitivity particularly for genes at lower expression levels.

Eliminating genomic DNA contamination is essential for accurate gene expression profiling by qPCR, which is particularly important for genes at low expression levels. Due to the presence of pseudogenes, even cross-intron primers are not a guarantee of avoiding amplification from contaminating genomic DNA. The Genomic DNA Control (GDC) in the PCR Array specifically detects potential genomic DNA contamination. A Ct for GDC less than 35 indicates the presence of genomic DNA contamination that may compromise the qPCR results.

B. First-strand cDNA synthesis

Use the same amount of total RNA in this reaction for every sample. High quality cDNA synthesis is vital for the following qPCR performance. We highly recommend using rtStar™ First-Strand cDNA Synthesis Kit (Cat# AS-FS-001), which is specifically optimized for and fully compatible with the Arraystar PCR Arrays.

> **NOTE:** *The RNA Spike-in in the kit is supplied as dried down. When using the kit first time, reconstitute the RNA spike-in by adding 50 μL Nuclease-free Water to the tube. Vortex and spin down. Leave on ice for 20 ~ 30 min to fully dissolve the RNA spike-in. Vortex and spin down again.*

1. Mix the following components in a 200 μL PCR tube for each sample. For multiple samples, calculate each component volume in the Table by multiplying with the number of samples. 10% extra volume is recommended for pipetting loss.

Oligo(dT)$_{18}$, or Random Primers	1.0 µL
dNTP Mix	1.0 µL
RNA Spike-in	2.0 µL
Template Total RNA	10.0 µL
Total volume	**14.0 µL**

2. Incubate in a thermal cycler at 65°C for 5 min, then immediately chill on ice for at least 1 min. Spin down briefly.

3. Add the following components directly to the product from STEP 2. The final volume will be 20 µL.

5 × RT Reaction Buffer	4.0 µL
RNase Inhibitor	1.0 µL
Reverse Transcriptase	1.0 µL
Total volume	**20. 0 µL**

4. Incubate at 25°C for 10 min, followed by 30 min at 45°C.

5. Terminate the reaction at 85°C for 5 min. Keep the finished First Strand cDNA Synthesis reaction on ice until the next step. OK to store at -20°C for later use.

C. Perform qPCR for the PCR array

1. Dilute the cDNA in Nuclease-free Water. If 1.5 µg input RNA is used with rtStar™ First-Strand cDNA Synthesis Kit (Cat# AS-FS-001), the dilution factor is 1:80. Mix well and spin down. The diluted cDNA is used as the qPCR template in Wells 1-379 (Table 1) for assays on the Transcripts, Housekeeping gene Internal Controls, and Spike-in External Controls.

2. For GDC Controls, combine 1 µL NRT (mock cDNA synthesis reaction without the reverse transcriptase) sample or 1 µL RNA sample (without cDNA synthesis), 5 µL SYBR Green Master Mix, and 4 µL Nuclease-free Water. Mix well and spin down.

3. For Blank Controls, combine 25 µL SYBR Green Master Mix and 25 µL Nuclease-free Water. Mix well and spin down.

4. Prepare the qPCR Mix according to the Table below. There are total of 384 wells of PCR reactions. Some extra volumes have been included for liquid dispensing loss.

Arraystar SYBR Green Master Mix	2010 μL
diluted cDNA template	1600 μL
ddH_2O	390 μL
Total volume	**4000 μL**

5. Load the 384-Well PCR Array:

   a. CAREFULLY peel off the original plate seal from the PCR Array;

   b. Add 10 μL aliquot of the cocktail from STEP 4 to each well (except the control wells P20-P24);

   c. Add 10 μL aliquot of the GDC Mix from STEP 2 to well P23 to detect genomic DNA contamination.

   d. Add 10 μL aliquot of the Blank Mix from STEP 3 to wells P20~P22 and well P24.

   e. CAREFULLY but tightly seal the PCR Array plate with an optical adhesive cover. Be sure that no bubbles present in any of the wells. To remove bubbles, tap the plate gently on the bench top or centrifuge the plate briefly.

   f. Keep the plate on ice while setting up the PCR program described in "Running Real-Time PCR Detection" below.

6. Run Real-Time PCR

Cycles	Temperature	Time
1	95℃	10 minutes
40	95℃	10 seconds
	60℃	1 minute
Melting curve analysis		

D. Data pre-processing and data analysis

Pre-process the qPCR run data according to the qPCR instrument manufacturer's instructions. If using baseline and threshold method for Ct calculations, such as with ABI 7900HT, the optimal baseline and threshold obtained by manual setting should be applied consistently across all assays on the plate, which is preferred over automatic software setting for each reaction for better reliability and accuracy.

Inspect the melting curves of the post-PCR products to verify the amplification specificity. If the melting curve has multiple peaks or poor peak morphology deviated from that of the known single amplicon product, it may

indicate non-specific off-target amplification or primer dimer formation, which will compromise the quantification. In such cases, gel electrophoresis or DNA fragment analysis can be performed to verify whether the product is in fact from a single correct amplicon.

Export the raw Ct values to Excel. Free, easy-to-use data analysis software is available online, please visit www.arraystar.com for detailed instruction. The data analysis procedures include the following steps:

Data pre-processing

1. Set all Ct values ≥ 35 or N/A (not detected) to 35. From this point on, any Ct value equal to 35 is considered a negative result.

2. Examine the Ct values of the Genomic DNA Control (GDC) wells. If the value is 35, no genomic DNA contamination is detected and no action is needed. If the value is less than 35, genomic DNA contamination is evident and the result may be compromised.

3. Before initiating the data analysis, the RNA spike-in wells are compared. Outlier samples may be identified and considered for exclusion in the further data analysis.

4. Inter-plate calibration (IPC) can be performed using the PPC assay replicates either with the data analysis software or manually. For each plate, verify that the replicates have Ct standard deviation ≤ 0.5. If this is not the case, exclude the outlier if it can be identified. Calculate the average of the replicates for each plate, the overall average (average of IPC values from all plates). The calibration factor for each plate is calculated as the difference between the plate average and the overall average:

$$calibration\ factor = IPC(plate\ n) - IPC(overall)$$

The Ct value is corrected with the calibration factor as:

$$Ct_{RNA} = Ct_{RNA}(Raw\ value, plate\ n) - IPC(plate\ n) + IPC(overall)$$

or

$$Ct_{RNA} = Ct_{RNA}(Raw\ value, plate\ n) - calibration\ factor$$

For example:

	Plate 1	Plate 2	Plate 3
GLUT1	20.26	20.83	20.44
IPC Plate average	16.70	16.70	16.40
IPC overall average	16.60	16.60	16.60
Calibration factor	0.10	0.10	-0.20
GLUT1 (Calibrated)	20.16	20.73	20.64

5. Calculate the ΔCt for each RNA in the plate.

$$\Delta Ct_{RNA} = Ct_{RNA} - average(Ct_{HKs})$$

Where *average* (Ct_{HKs}) is the geometric mean of the Ct values derived from the multiple HK genes. These most stably expressed housekeeping reference genes were selected from a broad range of samples by our stringent algorithm that evaluates the optimal properties and the number for endogenous controls.

■ Data analysis

1. Calculate the ΔΔCt for each RNA

$$\Delta\Delta Ct = \Delta Ct(sample\ 1) - \Delta Ct(sample\ 2), \text{ between samples}$$

or

$$\Delta\Delta Ct = \Delta Ct(group\ 1) - \Delta Ct(group\ 2), \text{ between group averages}$$

2. Convert ΔΔCt to fold change (FC)

$$Fold\ Change = 2^{-\Delta\Delta Ct}$$

NOTE: *By convention, if the fold change is greater than 1, the result is reported as a fold up-regulation. If the fold change is less than 1, its negative inverse (-1/FC) is reported as a fold down-regulation.*

3. When comparing profiling differences between two groups (such as disease versus control) with biological replicates, the statistical significance of the difference can be estimated as p-value by *t*-test. RNAs having fold changes ≥ 2 and *p*-values ≤ 0.05 are selected as the significantly differentially expressed RNAs.

   NOTE: *Fold change is related to biological effect size. Ranking by fold change is preferred over p-value. qPCR as commonly used in confirmation has a limit of quantification of 0.5 ΔCt, which is equivalent to approximately 1.5 fold change.*

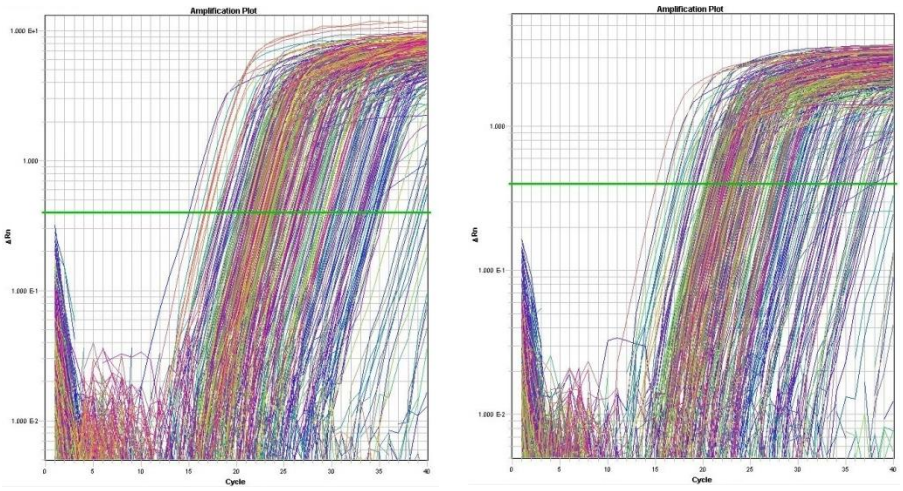
4. Other analyses such as scatter plots, volcano plots, and bar graph of expression differences for the mRNAs are performed and included in the full service standard analysis package.

III. Quality Control and Sample Data

A. PCR Array validation

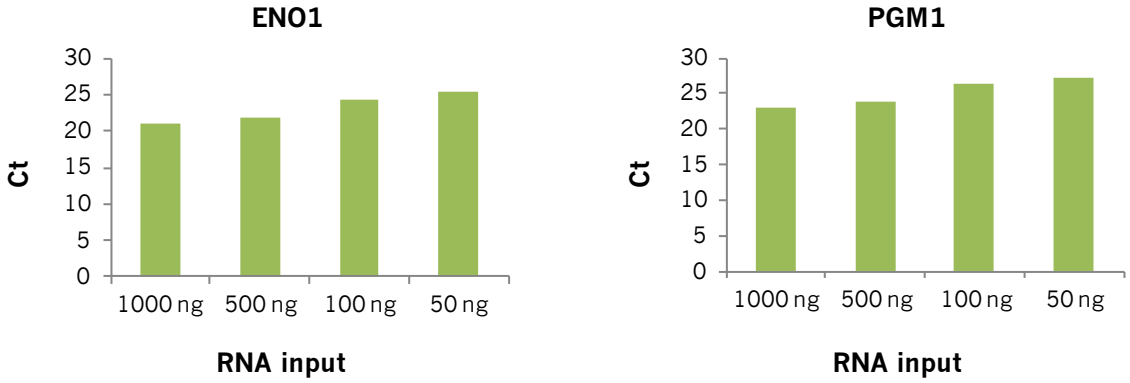
■ Real-time qPCR Validation

The performance of NuRNA™ Human Central Metabolism PCR Panel was tested using a cohort of 10 cell line samples. The extracted RNA samples were converted to cDNA using rtStar™ First-Strand cDNA Synthesis Kit. The cDNAs were profiled using the PCR array according to aforementioned protocol without modification. The results are charted on the amplification plots for the entire plate for two cell lines.



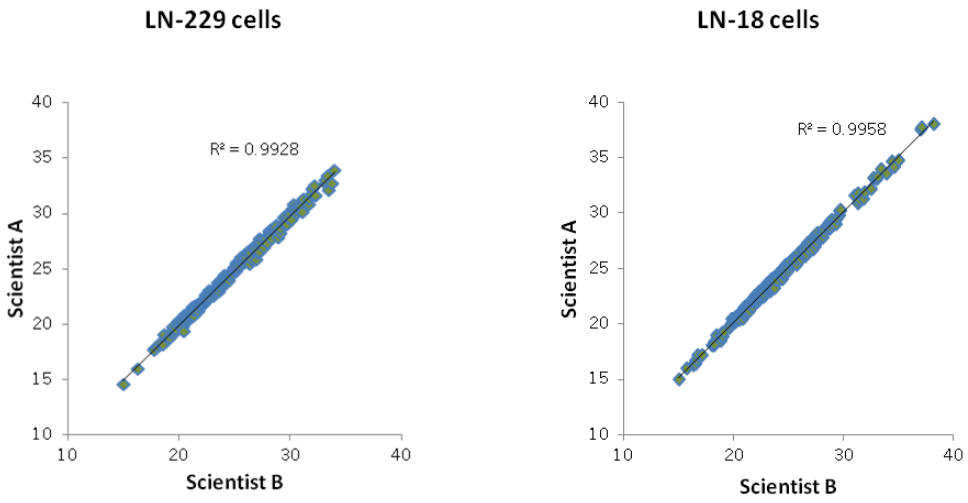
Sensitivity

The decreasing input amounts of total RNAs from human glioblastoma cell lines were converted to cDNAs and profiled by the PCR arrays. The Ct values were determined using the software default automatic baseline and Ct settings. ENO1 and PGM1 transcripts were detected at Ct values of 25.2 and 27.0 respectively, with the input RNA amount as low as 50 ng.



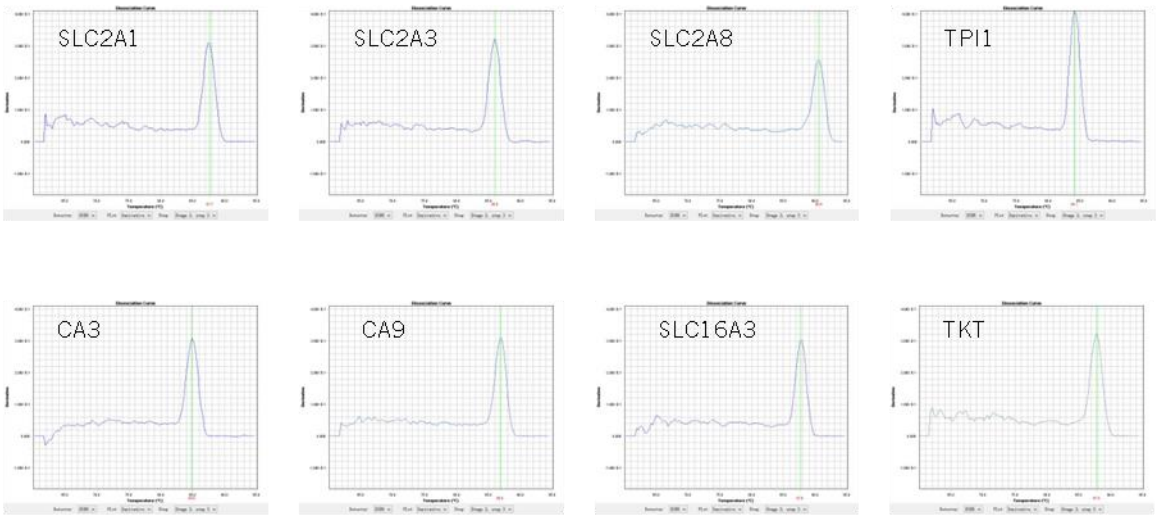
Reproducibility

Two independent runs of NuRNA™ Human Central Metabolism PCR Array were conducted by two different scientists A and B at two different times using two cell lines LN-229 and LN-18. The results demonstrate the high reproducibility with correlation R^2>0.98.



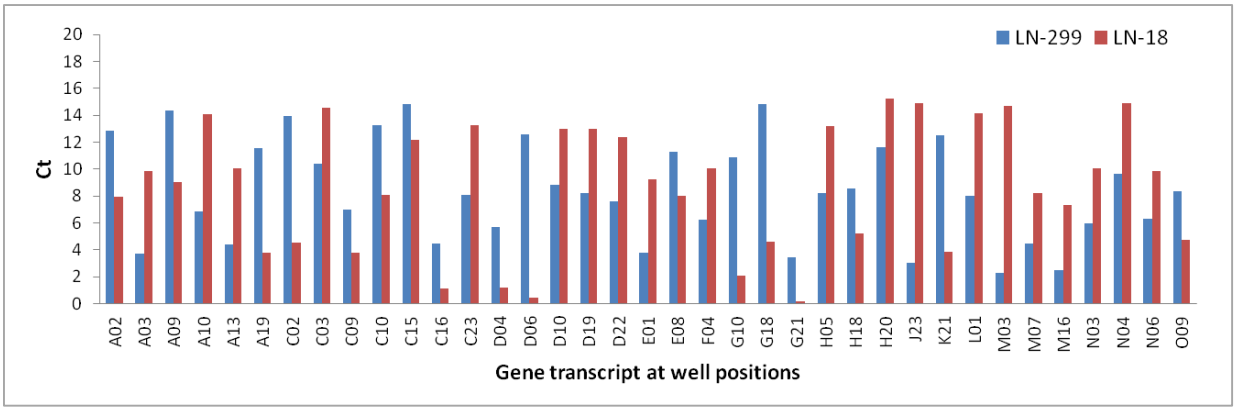
Specificity Test

The amplification products of solute carrier (SLC2A), carbonic anhydrase (CA), triosephosphate isomerase (TPI) and transketolase (TKT) transcripts and transcript isoforms were analyzed by melting curves. All showed single sharp melting peaks. The results demonstrate the high PCR amplification specificities on the array.



B. Sample data: Analysis of human central metabolism transcript levels in cell lines

The data were generated using RNA samples extracted from two glioblastoma cell lines LN-229 and LN-18. The normalization was carried out using the average of the housekeeping genes. ΔCt for the gene transcripts in the well positions are charted below.



IV. Troubleshooting

Problem	Possible solution
qPCR background too high	• Reduce the amount of cDNA used in the SYBR Green Master Mix.
No qPCR signals	• Inspect if the Internal Controls have valid qPCR signal. • Set SYBR Green as the Detector's Reporter Dye. • Use more cDNA in the Master Mix. • Lower the annealing temperature in Protocol STEP C.6 from 60°C to 50°C.
Baseline and threshold settings	• Follow the instructions of the qPCR system manufacturer. • Contact their technical support as necessary.

V. References

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VI. Terms and Conditions

By purchasing and using any part of the NuRNA™ Human Central Metabolism PCR Array, you agree to accept the following terms and conditions.

Product Use Limitation

Except as otherwise agreed in writing, all products are sold to end-users for research purposes only, and not for human or animal therapeutic or diagnostic use. We do not submit our products for regulatory review by any government body or other organization for clinical, therapeutic or diagnostic use. You are solely responsible for the way you use the products in compliance with applicable laws, regulations, and governmental policies.

The purchase of Product does not grant any right to use such Product in the practice of any methods covered by Arraystar intellectual property rights. You may not perform compositional, structural, functional or other analysis of our products, or undertake deconvolution or reverse engineering with respect to our products.

Product Warranty

Arraystar warrants that the Product will meet the specifications stated on the technical data sheet for that product, and agrees to replace the product free of charge if the product does not conform to the specifications. Notice for non-conformity and request for replacement must be given within 30 days of receipt of Products. In consideration of the above warranty by Arraystar, the buyer agrees to and accepts the following conditions:

That the buyer's sole remedy shall be to obtain replacement of the product from Arraystar; and

Arraystar Inc. shall not be responsible for replacing Product that has been improperly stored, handled, or used by buyer or End-User.

Arraystar, its Agencies and Representatives disclaim liability of any kind whatsoever, including, without limitation, liability for quality, performance, merchantability and fitness for a particular purpose arising out of the use, or inability to use the product. In no event shall Arraystar be liable for claims for any other damages, whether direct, incidental, foreseeable, consequential, or special (including but not limited to loss of use, revenue or profit), whether based upon warranty, contract, tort (including negligence) or strict liability arising in connection with the sale or the failure of products to perform in accordance with the stated specifications.

Arraystar disclaims any and all responsibility and liability for any injury or damage which may be caused by the failure of purchaser or end-user to follow said guidelines and specific product literature. It is the user's responsibility to determine and to adopt safety precautions as may be necessary.

VII. Technical Support

For additional information, manual download or technical assistance, please visit our website at www.arraystar.com, or contact us at:

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