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AVOID FALSE POSITIVE ELISA DATA:

IMPROVE PERFORMANCE WITH THE R&D SYSTEMS® QUANTIKINE® ASSAY

APPLICATION NOTE

INTRODUCTION

Enzyme Linked Immunosorbent Assays (ELISAs) are commonly used to quantify biomarkers in serum, plasma, and cell culture supernates. These samples contain a variety of proteins, blood components, and other factors that can interfere with the ELISA results, which is commonly referred to as a matrix effect. Eliminating these factors is essential to obtaining accurate sample values. Blocking reagents are commonly used in ELISA kits to reduce interference from proteins in samples which may produce false positive results. False positives can also occur from non-specific binding on the microplate. Solid phase blocking agents are specifically designed to saturate unoccupied binding sites on the ELISA plate surface and prevent this non-specific binding. R&D Systems uses specialized diluents that are specifically designed to alleviate false positives to give the most accurate results. Here are some kit-specific examples on how blocking reagents were used improve performance.

THE IMPORTANCE OF BLOCKING REAGENTS WHEN DETECTING HUMAN GDNF

Glial Cell Line-derived Neurotrophic Factor (GDNF) is a neurotrophic factor that has been shown to promote the survival of various neuronal subpopulations in both the central and peripheral nervous systems at different stages of their development. Neuronal subpopulations shown to be affected by GDNF include motor neurons, midbrain dopaminergic neurons, Purkinje cells and sympathetic neurons. GDNF is produced by Sertoli cells, type 1 astrocytes, Schwann cells, neurons, pinealocytes, and skeletal muscle cells. GDNF binding to GFR alpha 1 induces the recruitment of Ret, NCAM-1/CD56, various integrins, Syndecan-3, or N-Cadherin[1-3].

When developing the Human GDNF Quantikine ELISA (Catalog # DGD00), it was discovered that the serum sample values were not matching reported literature values using commercially available ELISAs[4-6]. These competitor assays were achieving values that ranged from non-detectable to > 600 pg/mL for serum samples from apparently healthy individuals, while the same samples were undetectable with the Quantikine ELISA.

FALSE POSITIVE DETECTION IN TWO COMMERCIALLY AVAILABLE ELISA KITS

Knowing that blocking agents alleviate non-specific binding, a further investigation into our competitors' assays was conducted by adding blocking agents to their diluents and testing them side by side with the diluent provided in their kit. As instructed in their respective inserts, serum samples were diluted 1:2 in Competitor A and 1:10 with acid treatment in Competitor B. Samples were tested neat on the Quantikine® ELISA. As shown in Table 1, none of the samples were detectable in using the Quantikine kit. However, competitor kits detected false positive GDNF levels ranging from 15.6 pg/ml to 692 pg/ml. Two of the samples were detectable with Competitor A using their diluent, but when blockers were added, the samples were no longer detectable. Four of the samples were detectable with Competitor B, but when blockers were added, the samples were no longer detectable.

TABLE 1 – FALSE POSITIVE HUMAN GDNF READINGS IN 2 COMPETITOR ELISA KITS

	QUANTIKINE	COMPETITOR A	COMPETITOR A + BLOCKERS	COMPETITOR B	COMPETITOR B + BLOCKERS
Standard Curve Range:	9.38 - 300 pg/mL	2.74 - 2000 pg/mL		15.6 - 1000 pg/mL	
Sample Dilution	Neat	1:2		(AT) 1:10	
Serum 1	*ND*	692	*ND*	44.4	*ND*
Serum 2	*ND*	614	*ND*	53.9	*ND*
Serum 3	*ND*	*ND*	*ND*	*ND*	*ND*
Serum 4	*ND*	*ND*	*ND*	18.2	*ND*
Serum 5	*ND*	*ND*	*ND*	*ND*	*ND*
Serum 6	*ND*	*ND*	*ND*	15.6	*ND*

ND = non-detectable, sample values << the lowest standard curve point

HUMAN GDNF SERUM VALUES ON R&D SYSTEMS ELISA VS COMPETITOR A ELISA.

We next determined whether removing blockers from the diluents in the Human GDNF Quantikine ELISA Kit would lead to false positive detection. The Competitor B ELISA kit was no longer commercially available, so they were not included in the additional testing. Eleven serum samples from apparently healthy individuals were tested on the Human GDNF Quantikine ELISA Kit according to protocol. Samples diluted in the Quantikine diluent were compared to identical samples in diluents that lacked blocking components. Identical samples were also tested using Competitor A's Human GDNF ELISA with our blocking reagents added to the diluent. U-118MG and U-87MG glioblastoma conditioned media supernate samples were also tested on both kits with and without our blocking reagents. These were used as a positive control[7] to ensure appropriate binding with and without blocking reagents

When using the Quantikine diluents as provided in the GDNF kit, no serum samples are detectable. When the blocking reagents are removed from the Quantikine diluents, 9 out of 11 serum samples were detectable, giving false positive sample values (Figure 1A). The U-118MG and U-87MG conditioned supernate samples are detectable whether blocking reagents are used or not, indicating that the blocking reagents are not suppressing the real GDNF detection in these positive controls.

When testing the ELISA kit from Competitor A, following the kit protocol and using the diluents provided, 9 out of 11 serum samples were detectable (Figure 1B). When blocking reagents were added to Competitor A's diluents, the serum samples were now undetectable. The U-118MG and U-87MG conditioned supernate samples are not detectable whether blockers are added to the diluents or left as provided in the kit. This indicates lower assay sensitivity with Competitor A when compared to the Quantikine ELISA, although the standard curve range is actually lower on Competitor A (Quantikine standard curve range: 9.38 – 300 pg/mL, Competitor A standard curve range: 2.74 – 2000 pg/mL).

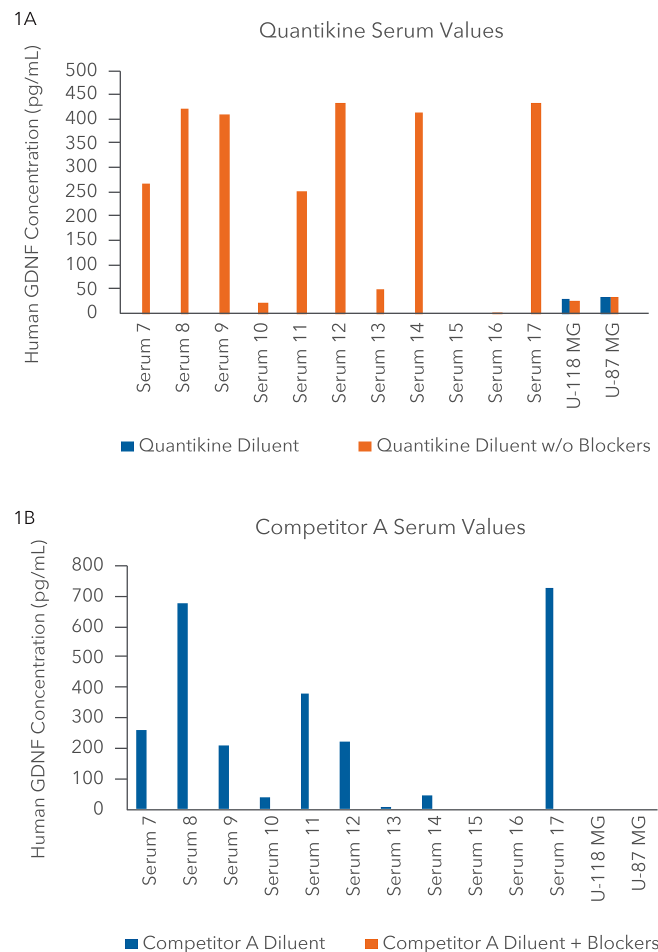

Figure 1. The importance of blocking reagents when detecting human GDNF. The absence of our proprietary blocking reagents resulted in false positives in 9 of 11 samples using the Quantikine ELISA (1A). Conversely, there were no false positive results in Competitor A's ELISA when our blocking reagents were added to the diluent (1B). Note that Competitor A lacked the sensitivity needed to detect GDNF in positive control supernates from U-118MG and U-87MG cell cultures.

TESTING THE IMPORTANCE OF BLOCKERS IN OTHER QUANTIKINE ELISA KITS

To extend our testing to demonstrate the importance of blocking reagents used in our Quantikine ELISA kits, some of the same serum samples were tested on two additional Quantikine ELISA kits with known non-detectable serum levels from healthy individuals. The Human TNF-α Quantikine ELISA Kit (Catalog # DTA00D) and Human IL-1β Quantikine ELISA Kit (Catalog # DLB50) were run according to kit instructions with the diluents provided and compared to using the same base diluents but with the blocking reagents removed. Helper T 1 (Th1) cell culture supernates were tested on each kit as a positive control and to ensure appropriate binding with and without blocking reagents.

HUMAN TNF-α QUANTIKINE ELISA

Tumor Necrosis Factor alpha (TNF-α), also known as cachectin and TNFSF1A, is the prototypic ligand of the TNF superfamily[8]. It is a pleiotropic molecule that plays a central role in inflammation, immune system development, apoptosis, and lipid metabolism[9-12]. TNF-α is also involved in a number of pathological conditions including asthma, Crohn's disease, rheumatoid arthritis, neuropathic pain, obesity, type 2 diabetes, septic shock, autoimmunity, and cancer[12-18]. TNF-α is not usually detectable in healthy populations, but increased serum levels are found under inflammatory and infectious conditions[12].

The Human TNF-α Quantikine ELISA detection limit is 15.6 pg/mL. Apparently healthy serum sample values are below the detection limit of this Quantikine ELISA[5]. Of the 7 serum samples, 5 samples were detectable when using the Quantikine diluent without blocking buffers (Figure 2). When using the Quantikine diluent as provided in the kit, none of the apparently healthy serum samples are detectable. Detection of the positive control Th1 cell culture supernate sample was detectable at similar levels with and without the blocking reagents, indicating appropriate specific binding is occurring.

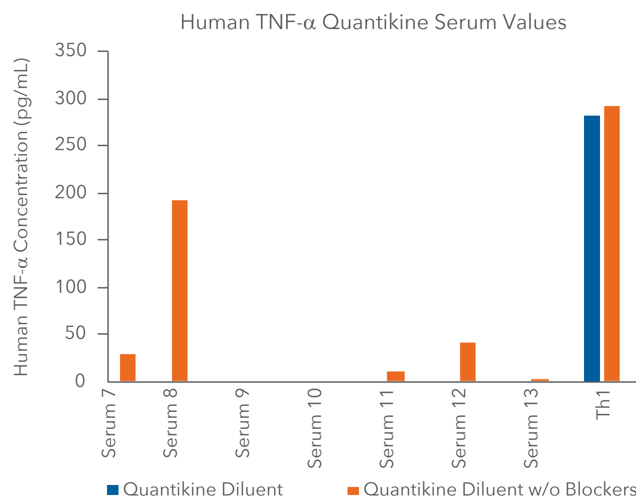

Figure 2. The importance of blocking reagents in the Human TNF-α Quantikine ELISA Kit. Seven serum and one cell culture supernate sample were tested in the Human TNF-α Quantikine ELISA Kit according to protocol, and with the blocking reagents removed. False positive results were observed when blocking reagents were omitted from the Quantikine diluent.

HUMAN IL-1β QUANTIKINE ELISA KIT

The Interleukin 1 (IL-1) family of proteins consists of the classic members IL-1α, IL-1β, and IL-1ra, plus IL-18, IL-33 and IL-1F5-F10. IL-1α and IL-1β bind to the same cell surface receptors and share biological functions[18]. With the exception of skin keratinocytes, some epithelial cells, and certain cells of the central nervous system, IL-1 is not produced by unstimulated cells of healthy individuals. However, in response to inflammatory agents, infections, or microbial endotoxins, a dramatic increase in the production of IL-1 by macrophages and various other cell types is observed. IL-1β plays a central role in immune and inflammatory responses, bone remodeling, fever, carbohydrate metabolism, and GH/IGF-I physiology. Inappropriate or prolonged production of IL-1 has been implicated in a variety of pathological conditions including sepsis, rheumatoid arthritis, inflammatory bowel disease, acute and chronic myelogenous leukemia, insulin dependent diabetes mellitus, atherosclerosis, neuronal injury, and aging-related diseases[20-23].

The Human IL-1β Quantikine ELISA detection limit is 3.91 pg/mL. Apparently healthy serum sample values are below the detection limit of this Quantikine ELISA. For the Human IL-1β Quantikine ELISA Kit, all 7 serum samples were detectable when using the Quantikine diluent without blocking reagents. When using the Quantikine diluents as provided in the kit, none of the serum samples were detectable, again showing that these blocking reagents are essential for preventing false positive sample values. The THP-1 cell culture supernate was detectable at similar levels with or without blocking reagents, indicating appropriate specific binding is occurring.

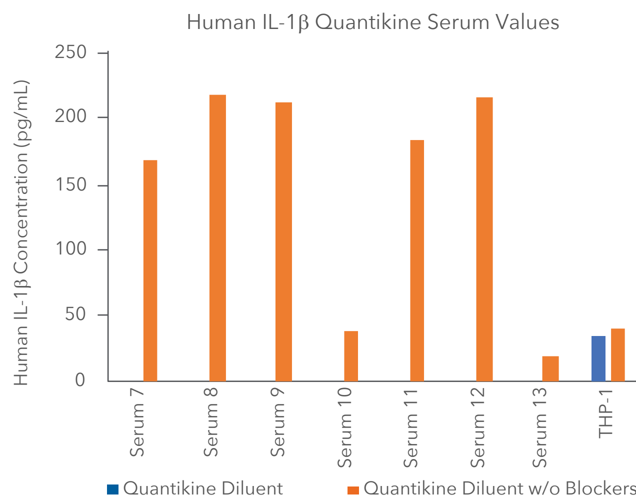

Figure 3. The importance of blocking reagents in the Human IL-1β Quantikine ELISA Kit. Seven serum and one cell culture supernate sample were tested in the Human IL-1β Quantikine ELISA Kit according to protocol, and with the blocking reagents removed. False positive Human IL-1β values are observed in Quantikine ELISAs when blocking reagents are omitted.

CONCLUSION

Manufacturing a quality ELISA kit begins with the reagents and the extensive optimization that takes place during the development of the assay. For R&D Systems ELISAs, antibodies, proteins, and diluents are developed and tested in-house and carefully selected to meet high performance criteria, stringent manufacturing guidelines, and quality control standards. Rigorous development and validation testing ensures the ELISA will perform with the ultimate precision, specificity, accuracy, and sensitivity.

Each sample type validated on an R&D Systems Quantikine ELISA has been fully optimized. Due to the complex matrix of some sample types, such as serum, non-specific binding can be a problem without such optimization, especially when little to no dilution of the sample is made. Conditioned media supernate samples have less of a problem with non-specific binding, which is why the sample values didn't change much when blockers were used in the diluents on the Quantikine assays. In order to get accurate testing results with your specific sample, you must use a fully optimized ELISA. The data presented above confirms R&D Systems Quantikine ELISAs are fully optimized to prevent non-specific binding from serum samples while some of our competitors are not. You get the most accurate results with our assays which is why R&D Systems ELISAs are the most trusted, most published ELISAs on the market.

CELL CULTURE METHODS

Human glioblastoma cancer cell lines (U-118MG and U-87MG) were cultured in base media supplemented with 10% FBS containing 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and maintained in a 5% CO_2 incubator at 37 °C.

Human monocytic leukemia cell line THP-1 was cultured in RPMI 1640 supplemented with 10% fetal bovine serum containing 100 µg/mL streptomycin and maintained at a 5% CO_2 incubator at 37 °C.

$CD4^+$ T cells were isolated from PBMCs (Ficoll separation) with MAGH102 (negative selection). Cells were cultured for 5-6 days in RPMI media supplemented with 10% FBS containing 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin sulfate, 20 ng/mL rhIL-2 (Catalog # 202-IL), 40 ng/mL rhIL-12 (Catalog # 219-IL), 5 µg/mL Ms x hIL-4 (Catalog # MAB304), 5 µg/mL Ms x hCD28 (Catalog # MAB342). Cell were then treated overnight with 10 ng/mL PMA + 500 ng/mL Ionomycin. The cells were maintained in a 5% CO_2 incubator at 37 °C.

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