bio-techne® | R&D SYSTEMS

ELISA Guide

A Clear and Easy Guide to ELISAs

Table of Contents

What is an ELISA?

ELISAs (Enzyme Linked Immunosorbent Assays) are a type of immunoassay that are commonly used to quantify levels of a specific target within a sample. Samples routinely used in ELISAs include serum, plasma, cell culture supernates, cell lysates, saliva, tissue lysates, and urine.

ELISAs are usually run in 96-well microplates coated with a capture antibody specific for the analyte of interest. Upon incubation with experimental samples, standards, or controls, the target analyte is captured by this antibody. A conjugated detection antibody binds to a different epitope on the target analyte. A substrate solution is subsequently added to produce a signal that is proportional to the amount of analyte bound. ELISAs can have different formats. Descriptions and diagrams of these can be found in the next section.

What's at the core of your immunoassay?

Your results matter, so what's inside your immunoassay should too. R&D Systems antibodies and proteins are the core of every Bio-Techne immunoassay platform. Our antibodies and proteins are highly specific, manufactured in-house to ensure reproducibility, and tested for suitability in every application we develop. In addition, the proteins used for the immunoassay standard and as antibody immunogens are typically full-length, recombinant proteins that are confirmed to be biologically active. This ensures that our standard closely mimics the natural protein and that the antibodies will recognize the native form of the analyte.

The Highest-Quality ELISAs Available

R&D Systems™, a Bio-Techne brand, has over 30 years of experience designing, testing, and optimizing immunoassay kits to ensure the highest level of performance in analyte quantification. We currently offer more than 600 complete, ready-to-use Quantikine ELISA Kits, 1,000 DuoSet ELISA Development Systems for numerous different analytes and species, including human, mouse, rat, canine, primate, and porcine, and an automated Simple Plex platform with over 200 assays available. Choosing quality reagents that will lead to results you can trust is one of the most critical aspects of scientific research.

- Most Referenced ELISA Manufacturer
- Flexible Formats Available
- Exceptional Analyte Selection
- Rigorous Validation Testing
- Extensive Quality Control Testing
- Long-term Consistency
- Bulk Packaging Available

Simple Reader™ Compact ELISA Plate Reader

Empower every lab member with their own compact, reliable microplate reader featuring 96 individual detection units and seamless plug-and-play functionality, perfect for any location.

- 4 LED Light Sources: 450 nm, 490 nm, 540 nm, 595 nm
- 90 Detection Units: Consistent and robust results
- Fast Read Time: Analyze data quickly with under 5 seconds per plate read

Learn More

Scan the QR Code or visit:
bio-techne.com/simplereader

ELISA Types

The four main types of ELISAs are direct, indirect, sandwich, and competitive. Each type of ELISA has its own advantages and disadvantages.

Direct ELISA

In a direct ELISA, an antigen or sample is immobilized directly on the plate and a conjugated detection antibody binds to the target protein. Substrate is then added, producing a signal that is proportional to the amount of analyte in the sample. Since only one antibody is used in a direct ELISA, it is less specific than a sandwich ELISA.

When to Use

Assessing antibody affinity and specificity

Investigating blocking/inhibitory interactions

Advantages

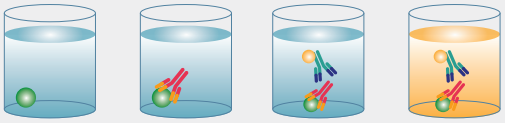
- Fast and simple protocol

Disadvantages

- Less specific as it uses only one antibody
- Potential for high background if all proteins from a sample are immobilized in well

Indirect ELISA

An indirect ELISA is similar to a direct ELISA in that an antigen is immobilized on a plate, but it includes an additional amplification detection step. First, an unconjugated primary detection antibody is added and binds to the specific antigen. A conjugated secondary antibody directed against the host species of the primary antibody is then added. Substrate produces a signal proportional to the amount of antigen bound in the well.



When to Use

Measuring endogenous antibodies

Advantages

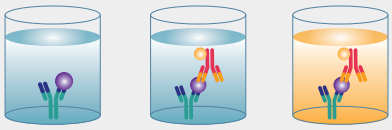
- Amplification using a secondary antibody

Disadvantages

- Potential for cross-reactivity caused by secondary antibody

Sandwich ELISA

Sandwich ELISAs are the most common type of ELISA. Two specific antibodies are used to sandwich the antigen, commonly referred to as matched antibody pairs. Capture antibody is coated on a microplate, sample is added, and the protein of interest binds and is immobilized on the plate. A conjugated-detection antibody is then added and binds to an additional epitope on the target protein. Substrate is added and produces a signal that is proportional to the amount of analyte present in the sample. Sandwich ELISAs are highly specific, since two antibodies are required to bind to the protein of interest.



When to Use

Determining analyte concentration in a biological sample

Advantages

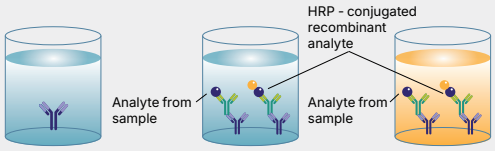
- Highest specificity and sensitivity
- Compatible with complex sample matrices

Disadvantages

- Longer protocol
- Challenging to develop

Competitive ELISA

Competitive ELISAs are commonly used for small molecules, when the protein of interest is too small to efficiently sandwich with two antibodies. Similar to a sandwich ELISA, a capture antibody is coated on a microplate. Instead of using a conjugated detection antibody, a conjugated antigen is used to compete for binding with the antigen present in the sample. The more antigen present in the sample, the less conjugated antigen will bind to the capture antibody. Substrate is added and the signal produced is inversely proportional to the amount of protein present in the sample.



When to Use

Determining concentrations of small molecules and hormones

Advantages

- Ability to quantitate small molecules

Disadvantages

- Less specific as it uses only one antibody
- Requires a conjugated antigen

Why use an ELISA over other techniques?

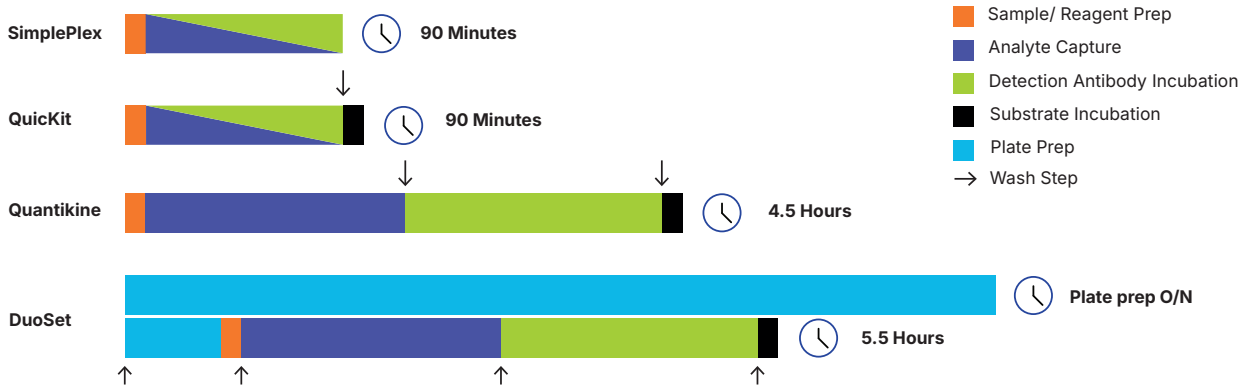
There are many different immunoassay platforms available to measure protein levels in biological fluids. ELISAs are preferred in many cases due to their sensitivity, specificity, accuracy, and ability to tolerate harsh buffers or pretreatments. Comparing an ELISA to a Western blot, sandwich ELISAs use 2 specific antibodies rather than one and allow for completely quantitative results, while a Western blot can see non-specific bands and are semi-quantitative at best. An advantage of ELISAs over different multiplexing platforms is the ability to customize the assay for the target analyte and not have to worry about interference caused by many other antibodies and proteins working together. The diluents used in our Quantikine ELISA kits are fully optimized to achieve the best performance for that analyte in complex sample matrices. The potential of observing cross-reactivity or interference is minimized and you can push the sensitivity limits with this technique.

ELISA Formats

Which Immunoassay is Right for You?

Kit	Quantikine ELISA	Quantikine QuicKit	Quantikine HS ELISA	DuoSet ELISA	Simle Plex Assay
Format	96-well Plate	96-well Plate	96-well Plate	Flexible	Cartridge
Benefit	• Most Published • Low CVs	• Fastest Plate-based ELISA	• Highest Sensitivity	• Economical • Largest Menu • Flexible	• Hands Free • Sensitive
Sample Volume	10-200 µL	50 µL	10-200 µL	100 µL	2.5 - 25 µL
Number of Analytes	1	1	1	1	Up to 8
Assay Time	3.5 hours	90 minutes	4-4.5 hours	20 hours	90 minutes
Pre-coated	Yes	Yes	Yes	No	Yes

ELISA Assay Timelines



The Citation Pack Leader: Quantikine™ ELISAs

Kit Components

- Pre-coated 96-well Microplate
- Conjugated Detection Antibody
- Calibrated Immunoassay Standard
- Assay Diluent
- Calibrator Diluent(s)
- Wash Buffer
- Color Reagent A and B
- Stop Solution
- Plate Sealers

Quantikine Colorimetric Sandwich ELISA Kits

Quantikine ELISA Kits are complete, ready-to-use kits that represent the gold standard in single analyte detection. Kits are available for measuring a wide range of molecules including cytokines, chemokines, growth factors, proteases, and more.

Quantikine QuicKit™ Colorimetric Sandwich ELISA Kits

QuicKit ELISAs allow for rapid quantitation of a target protein in only 90 minutes. With a shortened protocol and only one wash step, these ready-to-use kits allow you to accomplish more in your day, while getting the same quality of a Quantikine ELISA.

Quantikine HS (High Sensitivity) Colorimetric Sandwich ELISA Kit

Quantikine HS ELISA Kits are complete assays generally used when very low levels of the target protein are expected. These kits utilize distinct protocols to achieve their impressive levels of analyte detection. Two different amplification systems are available.

QuantiGlo™ Chemiluminescent Sandwich ELISA Kits

QuantiGlo ELISA Kits use a chemiluminescent substrate for analyte detection and require a luminometer for output reading. These kits have a broad dynamic range in comparison to standard colorimetric immunoassays.

Parameter Colorimetric Competitive ELISAs

Parameter ELISA kits are complete microplate-based assays designed to accurately measure the levels of small molecules using the competitive ELISA format.

Quantikine QuicKit Colorimetric Sandwich ELISA Kits

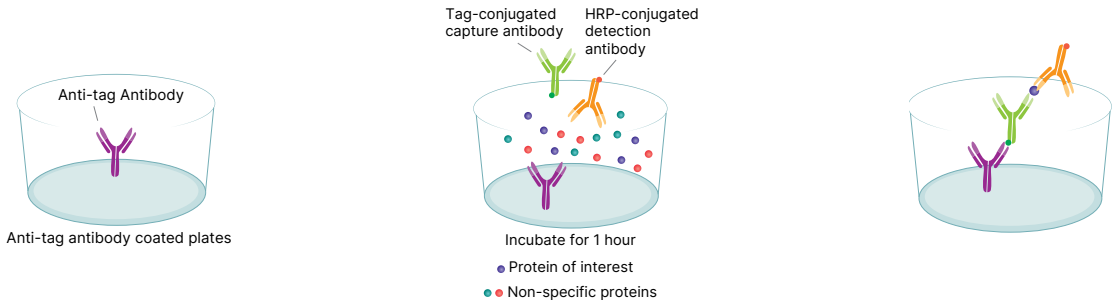
Accomplish more in your day without compromising quality. Quantikine QuicKit ELISAs provide quick, accurate quantitation of proteins in serum, plasma, and cell culture supernates. Unlike traditional ELISAs, QuicKit ELISAs have a fast, simplified protocol that only takes 90 minutes to results with just one wash step. You can expect the same high quality results, sensitivity, and lot-to-lot consistency as our existing gold-standard Quantikine ELISA kits.

How Quantikine QuicKit ELISA Assays Work

1 Add 50 µL sample
Add Antibody Cocktails

2 Wash Plate
Add Substrate

3 Incubate for 20 minutes
Stop and Read plate



Superior Linearity and Recovery

Linearity and recovery are key performance criteria to ensure your assay is accurately detecting samples, identifying false positives, and demonstrating your assay is ideal for your sample matrix. R&D Systems Quantikine ELISA Kits excel in this area and the Quantikine QuicKit ELISAs are no different.

Get Quantikine quality in a fraction of the time!

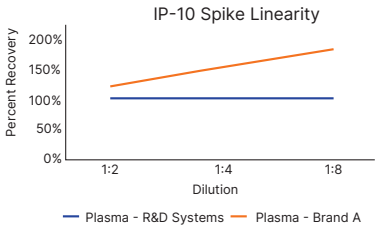


Figure 1. The R&D Systems IP-10 Quantikine QuicKit ELISA (Catalog # QK266) has superior linearity to the Brand A counterpart.

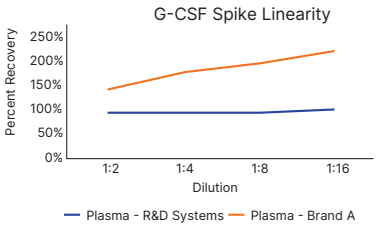


Figure 2. The R&D Systems G-CSF Quantikine QuicKit ELISA (Catalog # QK214) has superior linearity to the Brand A counterpart.

Learn More

Scan the QR Code or visit:
bio-techne.com/reagents/elisa-kits/quantikine-quickit-elisa

The DIY Solution: DuoSet™ ELISAs

Kit Components

- Capture Antibody
- Biotinylated Detection Antibody
- Mass-calibrated Standard
- Streptavidin-HRP
- Detailed Protocol

Accelerate your biomarker discovery and qualification with the full support of the R&D Systems ELISA development team.

- Novel Target ELISA Development
- New Sample Type Qualification
- New Species Qualification
- DuoSet Serum or Plasma Optimization
- Modification of Existing Kit Contents and Sizes

Learn More

Scan the QR Code or visit:
bio-techne.com/elisa-services

DuoSet Development Systems

DuoSet ELISA Development Systems contain the basic components required to develop a sandwich immunoassay for accurately measuring analytes in biological fluids. When complete kits are not an option, DuoSet ELISA Development Systems are an economical alternative to buying separate antibodies and protein standards. They are adaptable for use across multiple platforms and 384 well.

DuoSet IC (Intracellular) Assay Development Systems

DuoSet IC ELISA Development Systems are sensitive and convenient assays used to measure intracellular protein levels in cell lysates. R&D Systems DuoSet IC assays measure kinases, apoptosis-related and genotoxic stress proteins, heat shock proteins, and more. These signal transduction assays make an excellent alternative to Western blot, especially when used in combination with R&D Systems DuoSet IC Phospho-specific ELISAs. DuoSet IC Phospho-specific ELISAs are designed to measure the phosphorylation of a particular target analyte in cell lysates. Extensive validation work is done in-house to ensure specficity. These assays can be used in combination with DuoSet IC ELISAs that measure the levels of total protein.

DuoSet Supplemental Reagents

Complete your DuoSet kit with our supplemental products to run a full ELISA. Available in convenient kit packs or bulk sizes and for 96 and 384 wells.

Learn More

Scan the QR Code or visit:
bio-techne.com/reagents/elisa-kits/duoset-elisa-development-kits

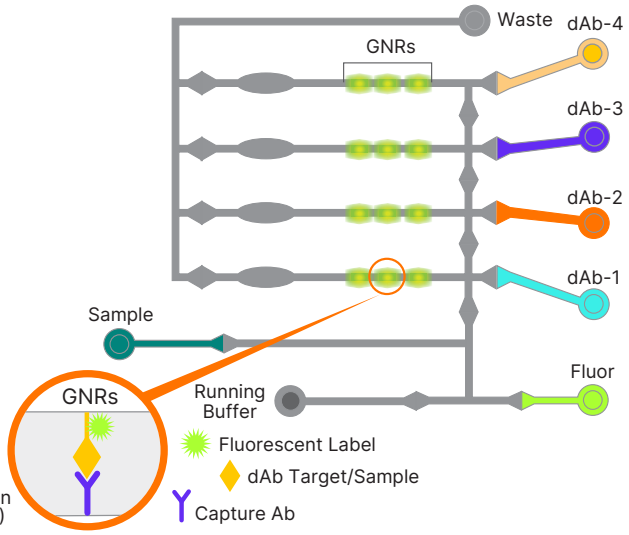
Your Next Generation ELISA: Simple Plex™ Assays

Simple Plex Assays

Simple Plex Assays on Ella bring your immunoassays to the next level. In just 90 minutes you get highly reproducible validated assay data with no manual steps. The assay performance behind that data includes sub-picogram level sensitivity, 4+ logs of dynamic range and reproducibility that rivals the best laboratory automation. The entire assay happens within the microfluidic cartridge which allow Simple Plex assays to achieve a high level of sensitivity, precision, and throughput.

How Simple Plex Assays Work

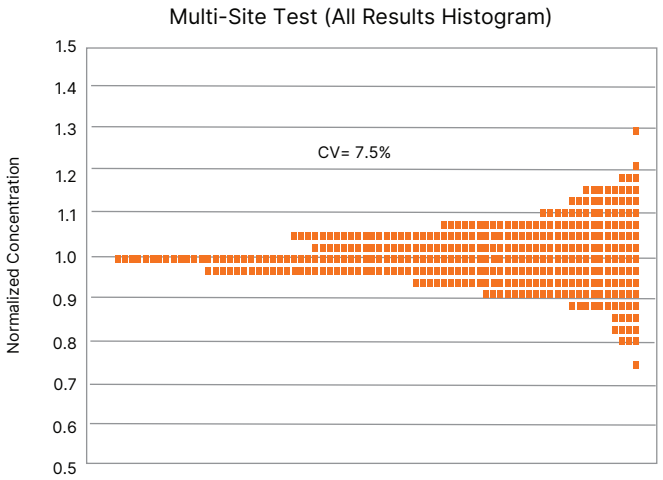
- Sample is routed through microfluidic channels
- Capture antibody captures target analyte
- Stringent wash removes unbound analyte
- Detection antibody migrates through the microfluidic channel
- Stringent wash removes unbound detection antibody
- Scan GNRs



Sandwich immunoassays happen in the Glass Nano Reactor (GNR)

Highly Standardized and Reproducible

Simple Plex assays give you single digit reproducibility — even across multiple users and labs. Simple Plex assays remove the manual steps from executing an immunoassay and enable a high level of precision and standardization even at very low analyte levels. This ensures that no matter where you measure a sample you get the same result. And because Simple Plex assays are correlated against our gold standard Quantikine ELISAs, it's easy to transfer to another platform whenever you need to.



Testing across 3 sites, 11 users and 9 Ella instruments. Four different assays were processed for CCL2, IL-6, TNF-α and VEGF A. Eight unique serum samples with 2 controls for a total of 704 answers.

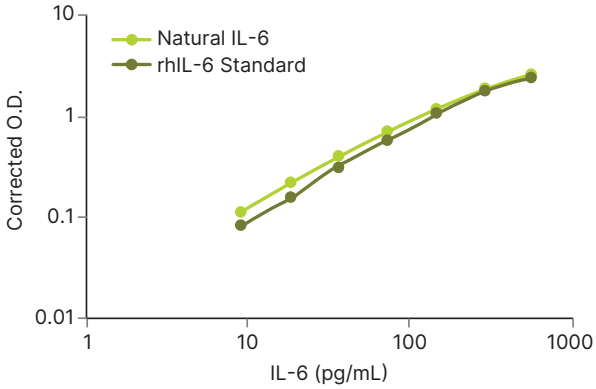
Learn More

Scan the QR Code or visit:
bio-techne.com/simple-plex

Ensuring ELISA Performance & Consistency

Accurate Detection of Natural Proteins

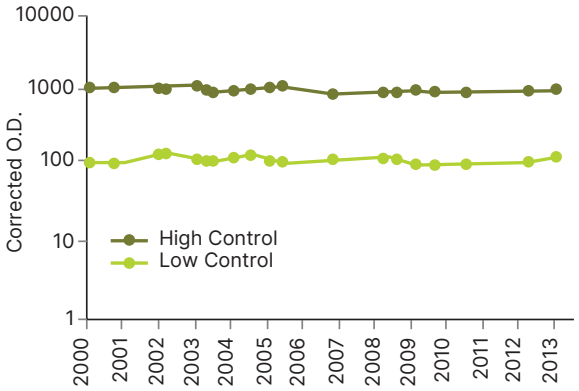
Antibody pairs recognize the supplied recombinant standard and the natural proteins in biological samples in a parallel manner, confirming that the kit can be used to measure the relative mass values of the natural analyte. R&D Systems has determined the ideal standard curve range for each assay, ensuring peak sensitivity and reproducibility of results.



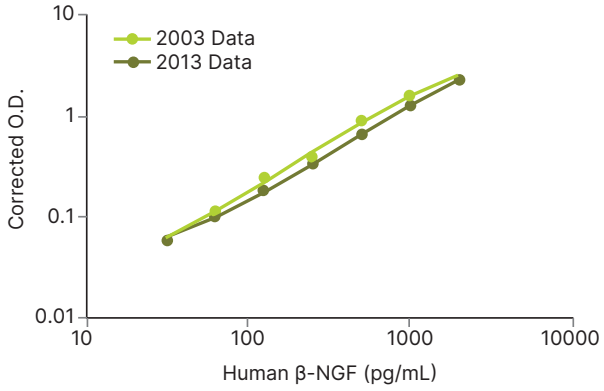
Recognition of Recombinant and Natural Human IL-6. Serial dilutions of rhIL-6 standard (dark green line) or natural IL-6 produced by unstimulated monocytes (light green line) were quantitated using the Human IL-6 DuoSet ELISA Development System (R&D Systems, Catalog # DY206). DuoSet ELISAs detect both recombinant and natural proteins in a parallel manner across a range of concentrations.

Confirmed Lot-to-Lot Consistency

All lots are tested to ensure low background, a linear standard curve, consistent assay sensitivity, and a broad dynamic standard curve range. Consistent standard curve O.D.s, control values, and natural sample values ensure that your samples run consistently over time.



Quantitation of Human β-NGF in High and Low Controls. Controls are assayed with every manufactured lot of Human β-NGF DuoSet ELISA Development System (R&D Systems, Catalog # DY256). Controls must read within a set range of ± two standard deviations from the mean. Controls for the β-NGF DuoSet ELISA Development System have remained consistent across 13 years.



Comparison of Human β-NGF Standard Curve O.D.s from 2003 and 2013. Using the Human β-NGF DuoSet ELISA Development System (R&D Systems, Catalog # DY256), standard curve values generated in 2003 and 2013 were compared for lot-to-lot consistency. Standard curve O.D.s remained consistent over ten years.

Precision & Reproducibility: Providing Confidence in Your Results

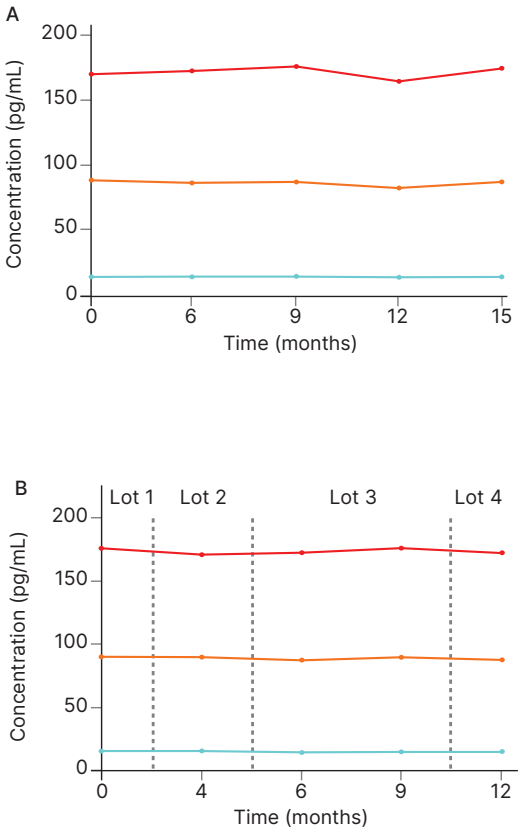
Immunoassay precision is defined as the reproducibility of results within and between assays.

This characteristic of an immunoassay is extremely important in order to:

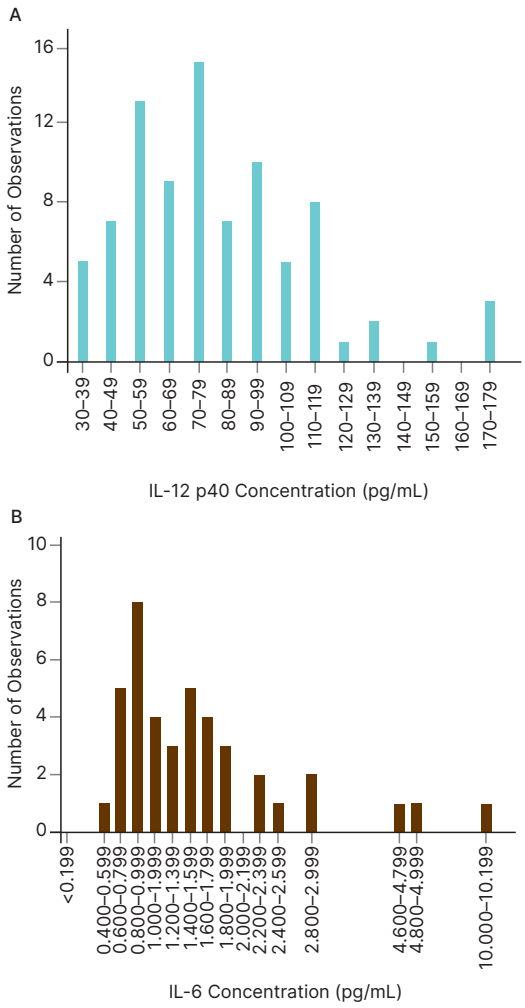
1. Provide assurance that the results obtained throughout a study are accurate and reproducible from one experiment to the next.

2. Determine if two results are the same or different.

Precision is measured as a coefficient of variation (CV) from the mean value. Two types of precision should be considered, intra-assay precision and inter-assay precision. Intra-assay precision is the reproducibility between wells within an assay. This allows the researcher to run multiple replicates of the same sample on one plate and obtain similar results. Inter-assay precision is the reproducibility between assays. Inter-assay precision guarantees that the results obtained will be reproducible using multiple kits over a period of time. R&D Systems Quantikine Immunoassays typically have CV values less than 10% across the standard curve for both intra- and inter-assay precision. These low CV values allow the researcher to perform repeated assays and be confident that the results are consistent throughout the study.



Quantikine ELISA Kits Are Tested for Stability and Reproducibility. A. Three samples with different concentrations of IL-6 (colored lines) were assayed using the same lot of the Human IL-6 Quantikine ELISA Kit (R&D Systems, Catalog # D6050B) over a 15 month period. **B**. Three samples with differing IL-6 concentrations (colored lines) were assayed using four different lots of the Human IL-6 Quantikine ELISA Kit (R&D Systems, Catalog # D6050B) over a 12 month period.



The Minimum Detectable Dose for Many Quantikine ELISA Kits Allows Proteins Present at the pg/mL Range to be Accurately Measured. A. Serum from 86 apparently healthy individuals was assayed using the Human IL-12/IL-23 p40 Quantikine ELISA Kit (R&D Systems, Catalog # DP400). **B**. Serum from 41 apparently healthy individuals was assayed using the Human IL-6 Quantikine HS ELISA Kit (R&D Systems, Catalog # HS600C).

Sensitivity: Measuring Proteins at the pg/mL Range

The minimum detectable dose is the lowest measurable value that is statistically different from zero. It is calculated by adding two standard deviations to the mean optical density value of several zero standard replicates and determining the corresponding analyte concentration from the standard curve. The better the sensitivity of an assay, the lower the useful working range (standard curve range) will be. Quantikine ELISAs are optimized to ensure high signal, low background, and the best sensitivity possible.

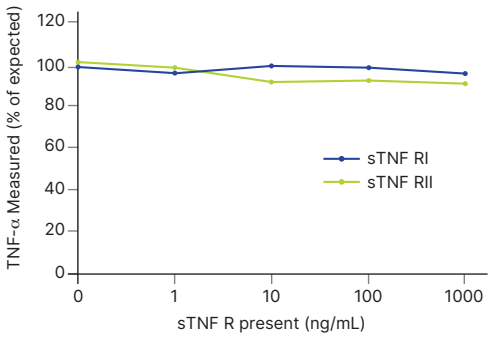
Linearity Experiments Identify False Positive Signals

False positive ELISA signals can be identified by assaying the linearity of dilution. Serial dilutions of a cell culture supernate were assayed for natural linearity using two different TIMP-2 ELISA Kits.

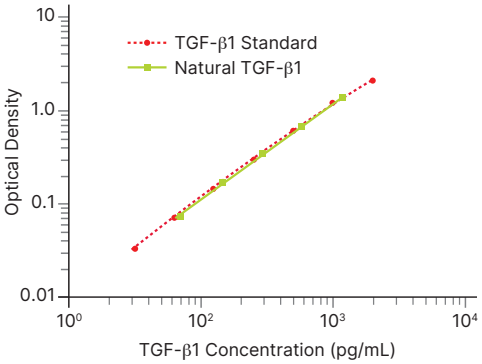
Diluted samples measured using the Human TIMP-2 Quantikine Kit (R&D Systems, Catalog # DTM200) gave recovery results between 105–108% of the neat sample, supporting the linearity claim of the kit. In contrast, the target analyte was not detectable beyond the first dilution in samples measured with the second kit, indicating that the assay was producing a false positive signal. ND=Not detectable.

Sample Dilution	Quantikine Kit	Competitior Kit
	Analyte Concentration Detected (ng/mL)*	
	4.16	**20.87**
1:2	105%	73%
1:4	108%	ND
1:8	106%	ND
Linearity Claim	85-115%	89-118%

* Samples were diluted prior to the assay as directed in the product data sheet. All samples and dilutions were within the standard curve range.



Interference Testing of the Human TNF-α Quantikine ELISA. TNF-α, at concentrations of 125–1000 pg/mL, was measured in the presence or absence of soluble TNF receptors (sTNF RI or sTNF RII) using the Human TNF-α Quantikine ELISA Kit (R&D Systems, Catalog # DTA00D). The results demonstrate that the presence of the soluble TNF receptors at concentrations up to 1000 ng/mL does not affect the TNF-α concentration determined using the Quantikine ELISA Kit.



Quantikine ELISA Kits Are Developed to Detect Natural and Recombinant Proteins. A serum sample containing activated human TGF-β1 was serially diluted (green line) and compared to the TGF-β1 standard curve (red line). Results show that the Human TGF-β1 Quantikine ELISA Kit (R&D Systems, Catalog # DB100C) measures recombinant and natural TGF-β1 with equal effectiveness.

Learn more about identifying and eliminating false positive results. Review our application note.

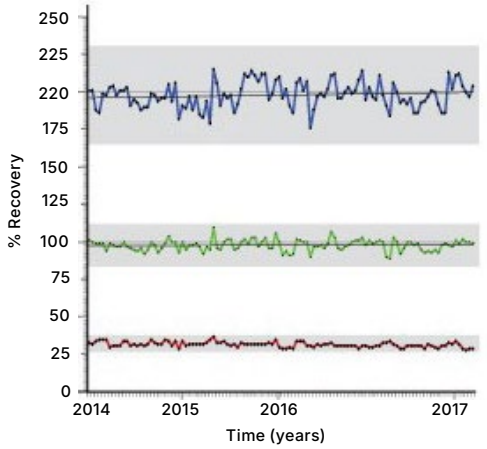
Scan the QR Code or visit:
bio-techne.com/resources/literature/avoid-false-positive-data-by-using-quantikine-elisas

What is the Importance of ELISA Controls?

The importance of including ELISA controls, both positive and negative, in your immunoassay helps to verify that the assay was run properly and everything is performing accurately.

Positive ELISA Controls

A positive ELISA control can be a recombinant or natural sample that you know will be detectable in the assay. Positive controls help to show that a negative sample is truly negative. The standard curve is one form of positive control and you can compare your results to the standard curve data that is provided in your product insert. R&D Systems also sells ELISA controls for the Quantikine ELISAs . Most Human Quantikine kits have a lyophilized tri-level control with expected ranges that are validated by our Quality Control and our Mouse/Rat Quantikine ELISAs include one control in the kit. These are great when running multiple plates or when you have multiple users running the assay, to verify that values are all within the expected ranges.



Quantitation of Human IL-6 in High, Medium, and Low Controls. High (blue line), medium (green line), and low (red line) controls are assayed with every manufactured lot of the Human IL-6 Quantikine ELISA Kit (R&D Systems, Catalog # D6050B). Controls for the Human IL-6 Quantikine® ELISA Kit fall within acceptable ranges (gray bars) and remain consistent from lot to lot.

ELISA Spike Controls

When using complex sample matrices, it is also important to make sure that there is nothing present in the matrix that interferes in the assay. It is recommended to spike in recombinant or natural protein into your matrix and verify that the amount you spike in is what you read out. For more information, refer to Section 4f. Our Quantikine ELISAs are already validated for many complex sample types, so refer to the kit insert for details.

Negative ELISA Controls

Negative controls help to verify that you are not obtaining any false positive results or non-specific binding. Use a sample that you know will express the protein you are measuring. If you are quantitating a cell culture supernate, a good negative control would be to test your cell culture media.

Sample Preparations

Sample Collection & Storage

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated. (Some proteins require the presence of fetal calf serum for stability.)

Plasma

Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Cell Culture Supernates

Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Cell Lysates

Solubilize cells in lysis buffer and allow to sit on ice for 30 minutes. Centrifuge tubes at 14,000 x g for 5 minutes to remove insoluble material. Aliquot the supernatant into a new tube and discard the remaining whole cell extract. Quantify total protein concentration using a total protein assay. Assay immediately or aliquot and store at ≤ -20°C.

Platelet-poor Plasma

Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 10 minutes at 2-8°C is recommended for complete platelet removal. Assay immediately or aliquot and store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Serum

Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Saliva

Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, assay immediately or aliquot and store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Urine

Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter. Assay immediately or aliquot and store at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Human Milk

Centrifuge for 15 minutes at 1000 x g at 2-8°C. Collect the aqueous fraction and repeat this process a total of 3 times. Assay immediately.

Tissue Homogenates

The preparation of tissue homogenates will vary depending upon tissue type. Rinse tissue with 1X PBS to remove excess blood, homogenize in 20 mL of 1X PBS and store overnight at ≤ -20°C. Perform two freeze-thaw cycles to break the cell membranes, and then centrifuge the homogenates for 5 minutes at 5000 x g. Remove the supernate immediately and assay. Alternatively, aliquot and store samples at ≤ -20°C.

Tissue Lysates

Rinse tissue with PBS, cut into 1-2 mm pieces, and homogenize with a tissue homogenizer in PBS. Add an equal volume of RIPA buffer containing protease inhibitors and lyse tissues at room temperature for 30 minutes with gentle agitation. Centrifuge to remove debris. Quantify total protein concentration using a total protein assay. Assay immediately or aliquot and store at ≤ -20°C.

Data Analysis: Calculation of Results

The values of the unknown samples are assigned in relation to the standard curve. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

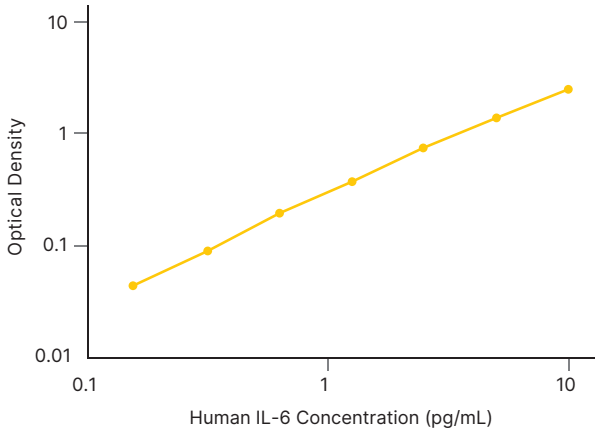
Always run ELISA samples in duplicate or triplicate. This will provide enough data for statistical validation of the results.

Average the duplicate or triplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.). The coefficient of variation (CV) of duplicates should be ≤ 20%.

Create a standard curve by reducing the data using computer software capable of plotting the mean absorbance (y axis) against the protein concentration (x axis). When possible, utilize the recommended data reduction method specified in the assay protocol.

If the recommended data reduction method is unavailable, it is recommended that various methods (e.g. linear, semi-log, log/log, 4 or 5 parameter logistic) be tried to see which curve best fits the data. One way to determine if the curve fit is correct is to back fit the standard curve O.D. values. To do this, first plot the standard curve. Next, treat standards as unknowns and interpolate the O.D. values from your standard curve. They should read close to the expected values (+/- 10%). Use the data reduction method that gives the best correlation value and back fit.

If software is unavailable, the data may be linearized by plotting the log of the concentrations versus the log of the O.D. on a linear scale.



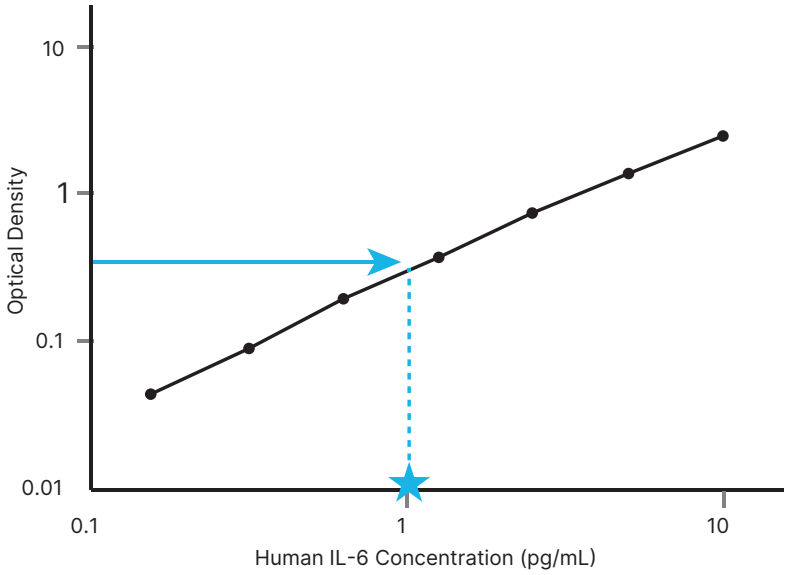
The best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

A representative standard curve is shown in the figure above from Human IL-6 HS Quantikine ELISA (R&D Systems, Catalog # HS006C).

(pg/mL)	O.D.	Average	Corrected
0	0.051 0.067	0.059	-
0:156	0.101 0.105	0.103	0.044
0.313	0.148 0.149	0.149	0.090
0.625	0.246 0.255	0.251	0.192
1.25	0.431 0.433	0.432	0.373
2.5	0.798 0.809	0.804	0.745
5	1.407 1.429	1.418	1.359
10	2.485 2.510	2.498	2.439

Calculating concentration of target protein in the sample

To determine the concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding concentration. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.



Calculating the coefficient of variation

The coefficient of variation (CV) is the ratio of the standard deviation to the mean, which is usually expressed as a percentage.

Calculating CV is important as it can indicate any inconsistencies or inaccuracies in your ELISA results. The CV of duplicates should be ≤ 20%. A larger CV indicates greater inconsistency and possible error.

General Protocol

for Running an R&D Systems Quantikine ELISA

Note: *Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.*

1. Prepare all reagents, working standards, and samples as directed in the Sample Preparation and Reagent Preparation sections of the kit booklet.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of the Assay Diluent to each well.
4. Add 100 µL of standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. ***Note:*** *Samples may require dilution. See the Sample Preparation of your kit booklet for dilution instructions.*
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of the target antigen Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without any correction may be higher and less accurate.

Calculation of Results

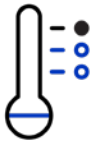
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the target analyte concentrations vs. the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Best Practices & Techniques

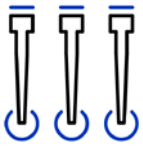
While R&D Systems builds Quantikine ELISA kits to be robust in the hands of even inexperienced users, there are several tips and tricks that can help even the experienced user get the most from their assay.



Make sure all reagents are brought to room temperature before using (unless instructed to keep them cold).

If you are not going to run the entire plate, ensure that the remaining strips are sealed in the plate bag with the desiccant to prevent moisture from degrading the plate.

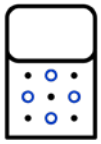
For standards that are not single use, it is best to aliquot the remaining standard into smaller volumes and freeze. This allows you to avoid repeated freeze-thaws.



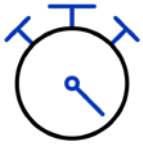
Multichannel pipettes speed the ability to plate your standard and samples and lead to more consistent results.

When pipetting, dispense liquid with the pipette tips held at an angle and not touching the bottom of the well.

While it is not necessary to change your pipette tips between each replicate, it is recommended that you change them between different samples or standards to prevent contamination.



It is highly recommended that a plate washer is used as manual plate washing can lead to higher backgrounds.



When washing plates, either manually or with a plate washer, be sure to give the wash buffer time to work by adding a 30 second soak time in between washes.

Pay close attention to the incubation times. As a general guide, the incubation time should not vary by more than +/- 5 minutes per hour of incubation time.

If the assay calls for incubation in a cold environment, at 2–8 °C, and you are running multiple assays, do not stack the plates on top of each other. Instead place them individually on the shelf.

Download Our ELISA Tips and Tricks Poster

Scan the QR Code or visit:
bio-techne.com/resources/literature/elisa-tips-and-tricks-poster

Quantikine ELISA FAQs

What is included in a Quantikine Kit?

Quantikine Kits are complete kits consisting of a precoated microplate, conjugated detection antibody, standard, diluents, substrate, stop solution, wash buffer, and plate sealers. They are fully validated ELISAs for the sample types listed in the specific datasheet. They have been exhaustively tested for superior quality.

How many samples can be assayed in a Quantikine kit?

Most Quantikine Kits will run the standard curve and 40 samples in duplicate. Please refer to the datasheet for details on each kit.

What samples can be tested in the kit?

Typically R&D Systems Quantikine Kits are validated for sera, two types of plasma, and cell culture supernate. However, the samples validated in an ELISA can vary from product to product. The product datasheet and product-specific web page states all sample types that have been validated for use with the ELISA kit. These are the only samples for which we can support the claims. References may exist for other sample types. See the “Citations” tab on the product-specific web page for any published references citing the use of the kit with an alternate sample type. Unclaimed sample types should be validated by the customer.

Has this kit ever been tested with my sample type?

Unfortunately, R&D Systems has not routinely tested many sample types such as tissue homogenates or bronchoalveolar lavage with our ELISA kits. This does not mean that the ELISA kit is not suitable for other sample types. One will need to perform a spike and recovery study to determine if an unvalidated sample type will work with a particular kit. To perform a spike and recovery experiment, one should divide a sample into two aliquots. In one of the aliquots, the user should spike in a known amount of the kit standard. A dilution series is performed comparing the spiked versus the unspiked sample. Generally, samples with expected recovery and linearity between 80–120% are considered acceptable. This method may be used to validate any sample type that has not been evaluated by R&D Systems. For a more detailed spike and recovery protocol, please contact Technical Service. Note: Acceptable ranges should be determined individually by each laboratory. Please see the Citations tab on the product-specific web page for peer-reviewed papers utilizing a wide range of
sample types.

Why can't I detect any of my samples?

You will be able to quantify samples down to the lowest point on the standard curve. In some cases, the standard curve doesn't go down low enough to detect normal samples. You can check the Sample Values section in your kit booklet to find out what kind of sample values we obtained from apparently healthy individuals. You may also want to review the literature to find out if there is an established normal range for your target. It is important to recognize that assay platforms and manufacturers differ in their calibrations for their unique assay products and reported measurements may not directly correlate.

Can I extend the standard curve (in either direction)?

R&D Systems cannot support kit results outside the stated range under any circumstances. A specific range was chosen because of confidence in the reproducibility of the assay.

Why doesn't the assay range extend to the stated sensitivity?

Sensitivity is the lowest measurable value that is statistically not equal to zero. It is calculated based on the signal of the background and the inherent variability of the assay. It is commonly determined by taking the mean O.D. plus two standard deviations from 20 zero replicates. This value is converted into analyte concentration from the standard curve. The low standard is the lowest possible point at which R&D Systems feels confident that the value is in the linear portion of the standard curve and, therefore, quantifiable. Values which are greater than the sensitivity can be distinguished as separate from the background or the noise of the assay, however the confidence level for reporting these values is lower than if the sample values fall within the standard curve range.

Why is a sample dilution necessary in some kits?

There are primarily two reasons for dilutions. In some assays most samples read above the standard curve, thus requiring a dilution for analyte levels to fall within the range of the assay. A second reason for dilution is to limit interference due to factors in complex matrices.

Won't addition of Assay Diluent cause further dilution of the sample?

Since the assay diluent is added to all wells, standards and specimens are treated equally. Therefore, sample concentration can be read from the standard curve without adjusting for this dilution.

Is there enough Calibrator Diluent for all of my sample preparations?

The kits are designed with enough calibrator diluent to ensure that the vast majority of samples fall within the indicated range of the assay. Should you find that there is not enough diluent provided in the kit to dilute your samples, you have at least two options. Option 1) Samples can be diluted in two steps. The initial dilution in culture medium and a final dilution, of at least 1:10, into the Calibrator Diluent provided in the kit. Option 2) For a nominal charge, you can purchase additional diluent provided the same lot included in the kit is still available. Contact Technical Service for more information.

My diluents appear to contain precipitate. Is this ok?

Due to saturating amounts of some buffer components, some of the RD1 Assay Diluents contain a light to heavy precipitate. In these instances, it will be noted in the specific protocol booklet. If it is not noted in the protocol booklet, please contact Technical Service.

The assay protocol specifies to use the shaker at 500 rpm. This is too fast for my shaker. Is this correct?

This is 500 rpm with a 0.12 orbit. If the plate shaker has a larger orbit, then 500 rpm will be too fast. R&D Systems recommends the ThermoFisher Model # 4625 microtiter plate shaker. Assays requiring shaker incubations have been optimized for performance with these shaker specifications only.

Are controls available for kits?

R&D Systems offers tri-level control sets for the Human Quantikine ELISA Kits (colorimetric), Quantikine HS ELISA kits (high sensitivity), and QuantiGlo ELISA kits (chemiluminescent). Please inquire for specific ordering information.

What is the stability of supplemental ELISA controls?

Controls are assigned an expiration date of 6 months from date of receipt. They are to be used once and discarded. If the lyophilized controls are stored properly, it is possible that they will remain stable for an extended period of time, although we have not conducted extended stability testing. The controls have not been tested for stability after reconstitution.

I used your recombinant protein as a control in the corresponding ELISA kit. Why am I seeing discrepancy in mass values?

First, a large dilution is required to place the recombinant protein on the standard curve range. Typically this is a dilution from µg/mL to pg/mL. Any dilution step can introduce inaccuracy and the larger the dilution step the greater the potential for error. Any pipetting error or mis-calibrated pipet can result in apparent over- or under-recovery. Second, R&D Systems immunoassays have been developed to measure a level of protein captured by one antibody and detected by a second antibody. This measurement is calibrated to standards established when the kit was initially developed. The protein determination of these initial standards became the Master Calibrators to which all new standards are formulated. This provides R&D Systems immunoassay kits with consistency between manufacturing lots. In general, we would expect +/- 25% recovery of the amount stated on the vial when using the Quantikine ELISA to determine a protein concentration. There may be slight differences in the immunologically recognizable mass between lots of protein, so the apparent concentration provided on the vial may vary from lot-to-lot when measured in the ELISA. If you are using proteins to make controls, it is better to value assign the mass based on measurement in ELISA and not use the mass on the vial when setting control levels.

Why must I use polypropylene tubes for standard curve dilutions in certain assays?

Certain proteins or analytes will bind to glass and polystyrene, but do not readily bind to the polypropylene tubes.

Why are my wells green after adding the stop solution?

This happens when the substrate in the well does not completely mix with the stop solution. After addition of the stop solution, tap the plate gently or place on a shaker until the mixture in the wells turns yellow.

Why is there brown precipitate in my wells after addition of the stop solution?

This is due to incomplete washing after the HRP-labeled detection antibody (or streptavidin-HRP) incubation. When HRP is present during the substrate and subsequent stop solution additions, an orange-brown or brown precipitate is observed. This may be remedied by the addition of a 30 second soak on each wash step followed by complete removal of all liquid in the wells.

What is a competitive ELISA?

In the competitive immunoassay approach, also termed labeled analyte technique, there exists a competition between the endogenous unlabeled antigen and an exogenous labeled antigen for a limited amount of antibody binding sites. Therefore, a decreasing signal indicates higher concentrations of the analyte being measured.

What is a sandwich ELISA?

A sandwich ELISA uses an immobilized capture antibody specific for the analyte of interest in a sample. After the analyte is bound to the immobilized antibody, a labeled secondary antibody specific for the analyte is used for detection. The analyte is “sandwiched” between the two antibodies. The sandwich ELISA is extremely sensitive, and the values obtained are quantitative when compared with a standard curve.

Can a partial Quantikine ELISA plate be used?

The Quantikine ELISA plates have removable strips of wells. Unused wells may be removed from the plate, returned to the foil pouch containing the desiccant pack, and stored at 2–8°C for up to one month.

Can I stop an assay at any point, extend an incubation time or change the suggested incubation temperature?

R&D Systems has optimized the assays for both incubation times and temperatures. Each kit has only been validated for the protocol described in the kit datasheet. We cannot guarantee the performance of our kits when the protocol has been altered in any way.

Can reagents from different kits be interchanged?

Assay Diluent(s), Calibrator Diluent(s), and substrate may be interchanged if they have the same part number AND lot number. R&D Systems does “whole kit QC” which means that we cannot support the use of reagents from other lots or sources being substituted into an assay. Plates and Conjugate cannot be interchanged under any circumstance.

Why do I need to use a 4-PL curve fit for generating my standard curve?

R&D Systems develops and QCs most of our Quantikine ELISA Kits using a 4-parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the concentrations versus the log of the O.D., and the best fit line can be determined by linear regression. This procedure will produce an adequate but less precise fit of the data.

Why am I seeing high variability between sample duplicates?

The two main reasons for high variability in an assay is related to pipetting & washing technique.

Troubleshooting your Quantikine ELISA

Problem	Possible Cause	Solution
No signal or low signal	Reagents added in incorrect order, or incorrectly prepared	Repeat assay
		Check calculations, standard reconstitution, etc.
	Standard has been damaged (if there is a signal in the sample wells)	Check that standard was handled according to directions. Avoid vortexing.
		Use new vial
	Incorrect incubation conditions	Check incubation conditions were for the specified length, at the appropriate temperature, and shaker specifications were met if required.
	Incorrect filters	Check specified signal and correction wavelengths in the protocol
	Incorrect Storage/Handling	Check that kit was stored properly according to conditions indicated on the box label
Too much signal – whole plate turned uniformly blue	Insufficient washing/washing step skipped – unbound peroxidase remaining	See washing procedure
	Substrate Solution mixed too early and turned blue	Substrate Solution should be mixed and used immediately
	Plate sealers or reagent reservoirs reused, resulting in presence of residual HRP. This will turn the TMB blue non-specifically	Use fresh plate sealer and reagent reservoir for each step
	Work surface cleaned with bleach	Residual bleach fumes can oxidize TMB and cause non-specific high signal
Standard curve achieved but poor discrimination between points (low or flat curve)	Plate not developed long enough	Increase Substrate Solution incubation time
		Use recommended time
	Incorrect procedure	Eliminate modifications, if any
	Improper calculation of standard curve dilutions	Check calculations, make new standard curve
	Insufficient washing	See washing procedure
		If using an automatic plate washer, check that all ports are clean and free of obstructions, add a 30 second soak step and rotate plate halfway through the wash
	Plate sealer reused	Use a fresh plate sealer for each step

Problem	Possible Cause	Solution
Poor Duplicates	No plate sealers used	Use plate sealers
	Insufficient washing	See washing procedures
		If using an automatic plate washer, check that all ports are clean and free of obstructions
	Variations in incubation temperature	Avoid incubating plates in areas where environmental conditions vary
	Variations in protocol	Adhere to the same validated assay protocol
	Variation in pipetting	Ensure all pipette tips are securely fastened and dispensing consistent volumes
		Establish use of either forward or reverse pipetting for entirety of the assay
	Improper shaker	Check that shaker orbit and speed meet specifications indicated in the kit insert. Any splashing on the plate sealer or foaming of liquid in the sample can also result in poor precision.
	Saliva contamination	Wear a mask to avoid contamination
Poor assay to assay reproducibility	Plate sealers reused	Use fresh plate sealer for each step
	Improper calculation of standard curve dilutions	Check calculations, make new standard curve
		Use internal controls
No signal when a signal is expected, but standard curve looks fine	No cytokine in sample or levels below assay range	Repeat experiment
		Reconsider experimental parameters
		Obtain fresh samples, minimize freeze-thaw cycles
		Use enzyme inhibitors
	Sample matrix is masking detection	Dilute samples at least 1:2 in appropriate diluent, or preferably do a series of dilutions to look at recovery
		If specified in the kit protocol, the assay may only recognize the sample after specific treatment. Follow any sample treatments specified in assay insert.
Samples are reading too high, but standard curve looks fine	Samples contain cytokine levels above assay range	Dilute samples further and run again
Very low readings across the plate	Incorrect wavelengths	Check filters/reader
	Insufficient development time	Increase development time
Green color develops upon addition of stop solution when using streptavidin-HRP	Reagents not mixed well enough in wells	Tap plate
Edge Effects	Uneven temperatures around work surfaces	Avoid incubating plates in areas where environmental conditions vary
		Use plate sealers
Drift	Interrupted assay set-up	Assay set-up should be continuous – have all standards and samples prepared appropriately before commencement of the assay
	Reagents not at room temperature	Ensure that all reagents are at room temperature before pipetting into the wells unless otherwise instructed in the antibody inserts

General Protocol

for Running an R&D Systems DuoSet ELISA

Plate Preparation

1. Dilute the Capture Antibody to the working concentration in PBS without carrier protein. Immediately coat a 96-well microplate with 100 µL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.
2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 µL of Reagent Diluent to each well. Incubate at room temperature for a minimum of 1 hour.
4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 µL of sample or standards in Reagent Diluent, or an appropriate diluent, to each well. Cover with an adhesive strip and incubate 2 hours at room temperature.
2. Repeat the aspiration/wash as in step 2 of the Plate Preparation section.
3. Add 100 µL of Detection Antibody, diluted in Reagent Diluent, to each well. Cover with a new adhesive strip and incubate 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of the Plate Preparation section.
5. Add 100 µL of the working dilution of Streptavidin-HRP to each well. Cover the plate and incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 2 of the Plate Preparation section.
7. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
8. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without any correction may be higher and less accurate.

Learn How to Adapt the DuoSet ELISA Kit Protocol to 384-well Plate Format

Scan the QR Code or visit:
bio-techne.com/resources/literature/adapting-duoset-elisa-kits-to-384-well-plates

DuoSet ELISA Assay Optimization

There are many parameters which influence the results obtained in an ELISA. These include: antibody quality and concentrations, incubation times, incubation temperatures, detection reagent quality and concentration, and substrate type and quality. For this section, it is assumed that all recommended reagents are being used.

Antibody concentration—the best way to determine the optimal capture and detection antibody concentrations is to perform a grid experiment. A grid experiment provides a method to test many antibody pair concentrations using only one plate. Antibody starting concentrations will vary depending on antibody type (monoclonal versus polyclonal) used for capture and detection, see Table 1. Refer to the product inserts for capture and detection antibody types as well as recommended starting concentrations.

TABLE // 01

Recommended antibody starting concentrations

	Monoclonal Capture/ Polyclonal Detection	Monoclonal Capture/ Monoclonal Detection	Polyclonal Capture/ Polyclonal Detection
Capture Concentration	1, 2, 4 and 8 µg/mL	0.5, 1, 2 and 4 µg/mL	0.2, 0.4 and 0.8 µg/mL
Detection Concentration	50, 100, 200 and 400 ng/mL	0.25, 0.5, 1 and 2 µg/mL	50, 100, 200 and 400 ng/mL

To form the grid, divide a 96-well plate into 4 quadrants. See Table 2 for an example of a monoclonal capture-polyclonal detection grid experiment. The 6 columns in each quadrant represent capture antibody concentrations, the 4 rows in each quadrant represent standard curve points, and each of the 4 quadrants represents a different detection antibody concentration. Each quadrant is a "mini-grid", identifying different capture antibody and standard concentrations at one particular detection antibody concentration. In the grid experiment in Table 2, each quadrant contains all the possible combinations of capture antibody at 1, 2 and 4 µg/mL and standard curve points of ø (Diluent stated on the product insert), 1000, 2000, and 4000 pg/mL, at one detection antibody concentration.

From the multiple combinations of antibody pair concentrations illustrated on the grid, select the concentrations that give the best signal to noise ratio. The ø standard points give the "noise" or the background value that can be expected at each of the antibody pair concentrations. The 1000, 2000 and 4000 pg/mL standard curve points give the "signal" resulting from each of the many antibody pair concentrations. Select the highest signal to noise ratio that still gives an acceptable background. A signal to noise ratio of at least 10 is excellent, but the ratio should be at least five.

TABLE // 02

Grid experiment for monoclonal capture-polyclonal detection assay

	50 ng/mL detection						100 ng/mL detection					
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
	1 µg/mL capture	1 µg/mL capture	2 µg/mL capture	2 µg/mL capture	4 µg/mL capture	4 µg/mL capture	1 µg/mL capture	1 µg/mL capture	2 µg/mL capture	2 µg/mL capture	4 µg/mL capture	4 µg/mL capture
A	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
B	1000 pg/mL standard	1000	1000	1000	1000	1000	1000 pg/mL standard	1000	1000	1000	1000	1000
C	2000 pg/mL standard	2000	2000	2000	2000	2000	2000 pg/mL standard	2000	2000	2000	2000	2000
D	4000 pg/mL standard	4000	4000	4000	4000	4000	4000 pg/mL standard	4000	4000	4000	4000	4000
E	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
F	1000 pg/mL standard	1000	1000	1000	1000	1000	1000 pg/mL standard	1000	1000	1000	1000	1000
G	2000 pg/mL standard	2000	2000	2000	2000	2000	2000 pg/mL standard	2000	2000	2000	2000	2000
H	4000 pg/mL standard	4000	4000	4000	4000	4000	4000 pg/mL standard	4000	4000	4000	4000	4000
	200 ng/mL detection						400 ng/mL detection					

Background <0.2 O.D. units. Factors that influence background include: blocking reagent, capture and detection antibody concentrations, detection system, incubation times, diluents and washing technique.

Curve height preferably above 1.0, usually between 1.0 and 3.0 O.D. units. Factors that influence curve height include: capture and detection antibody concentrations (see grid experiment in Table 2), incubation times and temperatures, detection system concentration, avidity of antibodies for antigens, pH, diluents and quality of reader.

Detection system assay sensitivity may increase with increasing detection reagent concentration or alternate detection system. However, this may result in higher background readings.

Dilution of serum and plasma samples serum and plasma samples may require a dilution of at least 2-fold in an appropriate buffer to overcome matrix effects. Empirically determine the dilution of the samples required to result in linearity of dilution. When diluting samples, remember that the diluent used for the standard curve should be the same as that used for samples. If samples are diluted, include the appropriate dilution factor when calculating results.

BSA bovine serum albumin, used as a blocking and carrier protein. Since different grades of BSA exist and may contribute to background, an ELISA grade BSA should be chosen and validated.

Incubation temperatures the sample and detection antibody incubations should be performed at room temperature. Sample incubation overnight at 4°C or 1 hour at 37°C may increase assay sensitivity, but may also increase the background.

Interfering substances it is important to be aware of the possible presence of interfering substances such as heterophilic antibodies or rheumatoid factors. Please refer to The Immunoassay Handbook, edited by David Wild, Nature Publishing Group, copyright 2001, for suggestions on how to control for these substances.

Reagent reconstitution and storage conditions reconstitution and storage instructions provided with each reagent must be followed to ensure proper reagent performance.

Sample preparation and storage while not every analyte has the same stability within a given matrix, there are general precautions which should be followed. Samples that are not used immediately after preparation should be stored in single use aliquots at -70°C. A -20°C freezer may be acceptable, depending on analyte, if it is a manual defrost freezer. It is best if the samples contain carrier protein. Multiple freeze-thaw cycles should be avoided.

Samples/standard volume use of a larger sample/standard size (200 µL per well vs. 100 µL per well) may increase sensitivity.

Substrate substrates can vary. However, choosing an alternate substrate will require additional assay condition optimization. Some substrates require a longer incubation time to get the curve to a reasonable height. If the substrate is functioning as expected, sensitivity may be enhanced by increasing incubation time. Monitor the plate as it is developing to avoid excessively high backgrounds. Typically, the incubation time ranges from 10 to 30 minutes. Use the correct filters required to read the appropriate wavelength for the substrate chosen. This information is available from the substrate vendor. Incubation times sensitivity may be increased with a longer incubation time at room temperature. Be aware that the top of the curve may flatten out and become unusable, limiting the assay range. Additionally, background may increase.

Use of a shaker at room temperature may increase sensitivity. Shakers may be used for some or all of the incubation steps. Incubation times should be determined empirically.

Washing follow instructions given in your ELISA Protocol. Insufficient washing can result in high coefficients of variation (CVs), high background and poor results.

Sensitivity varies for each antibody pair. Sensitivity is defined by reliable discrimination from the zero standard. Factors which influence sensitivity include: capture and detection antibody concentrations (refer to the grid experiment shown in Table 2), incubation times and temperatures, avidity of antibodies for antigens, sample/standard volumes, pH, diluents and wash buffer formulation. However, there is a limit to the sensitivity that can be achieved with each antibody pair.

Troubleshooting your DuoSet ELISA

Problem	Possible Cause	Solution
High Background	Insufficient washing	• See washing procedure
		• Increase number of washes
		• Add a 30 second soak step in between washes
	Too much streptavidin-HRP or equivalent	• Check dilution, titrate if necessary
	Insufficient blocking	• Check blocking solution calculations
		• Increase blocking time
	BSA impurities	• Use high-quality BSA and consider evaluating a different preparation of BSA
	Incubation times too long	• Reduce incubation times
	Interfering substances in samples or standards	• Run appropriate controls
	Buffers contaminated	• Make fresh buffers
No signal	Reagents added in incorrect order, or incorrectly prepared	• Repeat assay
		• Check calculations and make new buffers, standards, etc.
	Contamination of HRP with azide	• Use fresh reagents
	Not enough antibody used	• Increase concentration
	Standard has gone bad (if there is a signal in the sample wells)	• Check that standard was handled according to directions
		• Use new vial
	Buffer containing FCS used to reconstitute antibodies	• Re-qualify your reagents of choice
	BSA impurities	• Use high-quality BSA and consider evaluating a different preparation of BSA
	Capture antibody did not bind to plate	• Use an ELISA plate (not a tissue culture plate)
		• Dilute in PBS without additional protein
	Buffers contaminated	• Make fresh buffers
Too much signal—whole plate turned uniformly blue	Insufficient washing/washing step skipped – unbound peroxidase remaining	• See washing procedure
	Substrate Solution mixed too early and turned blue	• Substrate Solution should be mixed and used immediately
	Too much streptavidin-HRP	• Check dilution, titrate if necessary
	Plate sealers or reagent reservoirs reused, resulting in presence of residual HRP. This will turn the TMB blue non-specifically	• Use fresh plate sealer and reagent reservoir for each step
	Buffers contaminated with metals or HRP	• Make fresh buffers

Problem	Possible Cause	Solution
Standard curve achieved but poor discrimination between points (low or flat curve)	Not enough streptavidin-HRP	• Check dilution, titrate if necessary
	Capture antibody did not bind well to plate	• Use an ELISA plate (not a tissue culture plate)
		• Dilute in PBS without additional protein
	Not enough detection antibody	• Check dilution, titrate if necessary
	Plate not developed long enough	• Increase Substrate Solution incubation time
		• Use recommended time
	Incorrect procedure	• Go back to General ELISA Protocol; eliminate modifications, if any
	Improper calculation of standard curve dilutions	• Check calculations, make new standard curve
Poor Duplicates	Insufficient washing	• See washing procedure
		• If using an automatic plate washer, check that all ports are clean and free of obstructions, add a 30 second soak step and rotate plate halfway through the wash
	Uneven plate coating due to procedural error or poor plate quality (can bind unevenly)	• Dilute in PBS without additional protein
		• Check coating and blocking volumes, time and method of reagent addition. Check plate used
		• Use an ELISA plate (not a tissue culture plate)
	Plate sealer reused	• Use a fresh plate sealer for each step
	No plate sealers used	• Use plate sealers
	Buffers contaminated	• Make fresh buffers
Poor assay to assay reproducibility	Insufficient washing	• See washing procedures
		• If using an automatic plate washer, check that all ports are clean and free of obstructions
	Variations in incubation temperature	• Adhere to recommended incubation temperature
		• Avoid incubating plates in areas where environmental conditions vary
	Variations in protocol	• Adhere to the same protocol from run to run
	Plate sealers reused, resulting in presence of residual HRP which will turn TMB blue	• Use fresh plate sealer for each step
	Improper calculation of standard curve dilutions	• Check calculations, make new standard curve
		• Use internal controls
	Buffers contaminated	• Make fresh buffers

Problem	Possible Cause	Solution
No signal when a signal is expected, but standard curve looks fine	No cytokine in sample or levels below assay range	• Use internal controls
		• Repeat experiment, reconsider experimental parameters
	Sample matrix is masking detection	• Dilute samples at least 1:2 in appropriate diluent, or preferably do a series of dilutions to look at recovery
Samples are reading too high, but standard curve looks fine	Samples contain cytokine levels above assay range	• Dilute samples and run again
Very low readings across the plate	Incorrect wavelengths	• Check filters/reader
	Insufficient development time	• Increase development time
	Coated plates are old and have gone bad	• Coat new plates
	Capture antibody did not bind to the plate	• Use an ELISA plate (not a tissue culture plate)
		• Dilute in PBS without additional protein
	Buffer containing FCS used to reconstitute antibodies	• Re-qualify your reagents of choice
Green color develops upon addition of stop solution when using streptavidin-HRP	Reagents not mixed well enough in wells	• Tap plate
Edge Effects	Uneven temperatures around work surfaces	• Avoid incubating plates in areas where environmental conditions vary
		• Use plate sealers
Drift	Interrupted assay set-up	• Assay set-up should be continuous – have all standards and samples prepared appropriately before commencement of the assay
	Reagents not at room temperature	• Ensure that all reagents are at room temperature before pipetting into the wells unless otherwise instructed in the antibody inserts

ELISA Kit Offerings

Quantikine Kits

Species	# of Kits
Human	496
Mouse	144
Rat	52
Canine	14
Porcine	13
Multi-Species	7
Cynomologus Monkey	3
Viral	2
Rhesus Macaque	1

DuoSet Kits

Species	# of Kits
Human	726
Mouse	280
Rat	55
Canine	16
Porcine	13
Equine	8
Feline	8
Primate	6
Rabbit	5
Multi-Species	5
Bovine	4
Guinea pig	3
Cynomolgus Monkey	2
Rhesus Macaque	2
Cotton Rat	1
Goat	1
Viral	1
C. botulinum	1

Supplemental ELISA Development Products

Product	Catalog #
DuoSet ELISA Ancillary Reagent Kit 1	DY007B
DuoSet ELISA Ancillary Reagent Kit 2	DY008B
DuoSet ELISA Ancillary Reagent Kit 3	DY009B
Clear Microplates, 25 Pack	DY990
Black Microplates, 25 Pack	DY991
ELISA Plate Sealers	DY992
ELISA Plate-Coating Buffer	DY006
Glo Substrate Reagent Pack	DY993
Stop Solution	DY994
Reagent Diluent Concentrate 1	DY997
Reagent Diluent Concentrate 2	DY995
Reagent Diluent Concentrate 3	DY004
Reagent Additive 1	DY005
Streptavidin-HRP	DY998
Substrate Reagent Pack - 8 vials color A, 8 vials color B	DY999
Substrate Reagent Pack - 16 vials TMB ELISA Substrate	DY999B
Wash Buffer Concentrate	WA126
Sample Diluent Concentrate 1 (5X)	DYC001
Sample Diluent Concentrate 1 (2X)	DYC002
EvenCoat™ Goat Anti-Mouse IgG Microplates, 5 Pack	CP001
EvenCoat™ Goat Anti-Mouse IgG Microplates, 15 Pack	CP002
EvenCoat™ Streptavidin Coated Plates, 5 Pack	CP003
EvenCoat™ Streptavidin Coated Plates, 15 Pack	CP004
Sample Activation Kit 1	DY010
Cell Lysis Buffer 1	890713
Cell Lysis Buffer 2	895347
Cell Lysis Buffer 3	895366
Cell Lysis Buffer 5	895890
Lysis Buffer 6	895561
Lysis Buffer 16	895935
Lysis Buffer 17	895943

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