Why Perform Spike/Recovery and Linearity Testing?

The sample value obtained from an ELISA is dependent upon the interaction between the protein of interest and the ELISA's antibodies, and comparison of this interaction relative to a recombinant protein standard curve. Buffer components, sample matrix, complement, rheumatoid factor, heterophilic antibodies and other factors can impact antibody binding in the natural sample and therefore influence the accuracy of ELISA results.

During ELISA assay development, two tests are commonly performed to determine if the value obtained from a sample is accurate or if there is some factor in the sample matrix interfering with measurement.

In Spike/Recovery assays, a known amount of recombinant protein is "spiked" into a sample and run in the ELISA. The resulting concentration, or "recovery" of the spiked material, demonstrates if the expected value can be measured accurately. If the recovered value differs significantly from the amount expected, this can be a sign that some factor in the sample matrix may be causing a falsely elevated or falsely depressed value.

As a simple example, if a serum sample is spiked with a solution with a known concentration of 1000 pg/ml recombinant human IL-2, but the spiked sample measures in the ELISA at 80 pg/ml, some factor in the serum sample may be inhibiting detection of IL-2 by the antibodies used in the assay.

In Linearity assays, a spiked or unspiked sample is serial-diluted, such as 1:2, 1:4, and 1:8. If a sample does not exhibit linear dilution, this indicates that a matrix component is interfering with accurate detection of a specific analyte at a given dilution. Sometimes, matrix interference will only occur if a factor is present at concentrations above a certain threshold, and if the sample is diluted, interference is no longer observed. If interference was observed during the Spike/Recovery testing, sometimes the corrective action is to dilute the sample to the point where interference is no longer observed. Some sensitivity may be sacrificed by performing such a sample dilution.

If an unvalidated sample type is to be tested using an R&D Systems ELISA assay, the following protocol can be used as a general guide for testing spike/recovery and linearity.

ELISA kits available from R&D Systems

Quantikine® ELISAs
R&D Systems Quantikine ELISA Kits contain a complete ELISA consisting of a pre-coated microplate, conjugated detection antibody, standard, diluents, substrate, stop solution, wash buffer, and plate sealers. Quantikine ELISAs are exhaustively tested for superior quality and consistent lot-to-lot performance. These kits are considered fully validated and "ready to use" for most common sample types such as serum, plasma, cell supernates, and often other sample type such as urine, tissue homogenates, saliva or milk. Refer to the product insert for a listing of sample types validated in each specific kit. Other sample types not listed in the product insert, such as BALF (broncheoalveolar lavage fluid) or CSF (cerebrospinal fluid) need to be validated by the researcher prior to measurement in the Quantikine ELISA. Note that Quantikine HS and QuantiGlo ELISAs also fall under this category.

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DuoSet® ELISA Development Kits

R&D Systems' DuoSets are designed for customers who have sufficient expertise to develop a useful ELISA, and who do not require the performance characteristics of a Quantikine ELISA. R&D Systems development of DuoSets includes testing for appropriate antibody pairing, reagent stability, parallelism using naturally-derived protein (if available), cross-reactivity/interference of related proteins, and lot-to-lot consistency. The diluent and assay protocol suggested by the DuoSet package insert will be suitable for most cell supernate samples. The measurement of other sample types, such as serum, plasma, and tissue homogenates, is not guaranteed and needs to be validated by the researcher prior to use in the DuoSet.

Luminex Assays

R&D Systems also offers Luminex Performance and Screening Assays. Spike/recovery testing can also be performed for validation of a sample in Luminex. If performing Spike/Recovery testing of Luminex assays, one can generally follow the protocol described for Quantikine ELISA testing.

I. Items required for Spike/Recovery testing

- DuoSet and ancillary reagents or Quantikine ELISA
- Concentrated Spiking Stock Solution
- 2.0 mL of a well-mixed sample with a value within the standard curve range

(If the neat sample measures above the standard curve range, dilute it until the value reads within the standard curve range. Use this dilution for the spike/recovery and linearity assays. If sample value is unknown, preliminary testing of a serial-dilution of the sample, such as neat, 1:10, and 1:20, should be run to establish sample value, prior to performing spike/recovery and linearity tests.)

II. Spiking Stock Solution Preparation

Many DuoSets include standards that are stored at much higher concentrations than their working concentrations. We recommend the spiking stock solution be at a concentration approximately 10-times the recommended high standard concentration. For example, if the ELISA protocol states the high standard will be 2000 pg/ml, prepare a spiking stock solution at a concentration of 20 ng/ml.

Quantikine ELISA standards may or may not be supplied as a concentrate. In cases where the reconstituted ELISA standard is used as the high point of the standard curve, contact R&D Systems Technical Service to order a second vial of standard to use as spiking stock solution. Reconstitute the standard using the diluent specified in the product insert.

Notes: Recombinant protein other than the ELISA standard can be used to generate a spiking stock solution, but using the ELISA standard as a spiking stock will eliminate the need to first mass assign recombinant protein being used as a spiking stock.

10x spiking stock is a general recommendation. The concentration of the sample after spiking should fall in the middle-to-high end of the standard curve range indicated in the protocol. Depending on anticipated sample values, it may be necessary to prepare a different spiking stock solution.

III. Standard curve preparation

A standard curve can be prepared by diluting the standard in reagent diluent per the kit insert's recommendation.

It is important that the diluents selected for reconstitution and for dilution of the standard reflect the environment of the samples being measured. For cell lysates, a buffer similar to the cell lysis buffer may be compatible. For serum or plasma, we generally suggest starting with PBS supplemented with 10 - 50% fetal calf serum.

Notes: Quantikine ELISAs typically include one or two Calibrator Diluents, which are used to make the standard curve and to dilute samples as needed. Generally speaking, RD5 Calibrator Diluent should be used for low protein content samples such as urine, saliva and cell supernates. RD6 Calibrator Diluent should be used for high protein content samples such as serum or plasma. However, for unvalidated sample types, the suitability of each Calibrator Diluent should be determined empirically.

The diluent suggested in the DuoSet package insert is suitable for most cell supernate samples. If assaying sample types other than cell culture supernates, each laboratory should develop and validate its own diluent. This altered diluent should only be used to dilute the standard curve. Do not use a serum-containing diluent to dilute the Detection Antibody or the Streptavidin-HRP.

IV. Sample and Control Spike Preparation

1. Label 3 tubes: unspiked, spiked, and control.
2. From a well-mixed sample, prepare two aliquots:
 a. Pipette 1.0 mL into a tube labeled "unspiked." This is the "unspiked" sample.
 b. Pipette 0.98 mL into a tube labeled "spiked." This will be used to generate the "spiked" sample.
3. Pipette 0.98 mL reagent diluent into tube labeled "control." This will be used to generate the "control spike."
4. Pipette an identical volume of Spiking Stock Solution into your "control" and "spiked" sample (i.e. add 20 uL of Spiking Stock into 980 uL "spiked" sample and 980 uL "control.") Prepare these samples using the same pipette, but take care to change pipette tips between spikes.

Note: The suggested volumes are based on needing 200 uL per well of sample, run in duplicates. If the assay procedure calls for smaller sample volumes or duplicates are not being run, a smaller volume can be used to prepare spiked samples and controls.

5. Vortex samples briefly. Avoid foaming.

V. Preparing Sample/Control Spike Serial Dilutions (Testing Sample Linearity)

To test samples for linearity, make serial dilutions of the Sample Spike and Control Spike. If the unspiked sample has a value greater than 60% of the high standard, test the sample for natural linearity using the same dilution series described below.

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1. <u>1:2 dilution</u>
Add 0.5 mL of Sample Spike, Control Spike, or unspiked sample to 0.5 mL Standard Curve Diluent.

2. <u>1:4 dilution</u>
Add 0.5 mL of 1:2 dilution to 0.5 mL Standard Curve Diluent.

3. <u>1:8 dilution</u>
Add 0.5 mL of 1:4 dilution to 0.5 mL Standard Curve Diluent.

These dilutions will be read off the standard curve to determine if dilutions of unvalidated samples are parallel to the standard curve and if the values of the sample dilutions are accurate.

The plate could be set up as follows:

	1	2	3	4	5	6
A	Standard 1		Spiked Sample (Neat)		Control Spike (Neat)	
B	Standard 2		1:2 Spiked Sample		1:2 Control Spike	
C	Standard 3		1:4 Spiked Sample		1:4 Control Spike	
D	Standard 4		1:8 Spiked Sample		1:8 Control Spike	
E	Standard 5		Unspiked Sample (Neat)			
F	Standard 6		1:2 Unspiked Sample			
G	Standard 7		1:4 Unspiked Sample			
H	Standard 8 (Blank)		1:8 Unspiked Sample			

Note: If changing Standard Curve diluent, we recommend running the Standard Curve both in the diluent recommended by the ELISA product insert and in the new diluent side-by-side to verify the change in diluent does not impact standard curve performance.

IV. Calculations

1. Spike/Recovery
% Recovery = <u>Observed - Unspiked </u> x 100
                      Expected

Observed = Spiked sample value
Unspiked = Unspiked sample value
Expected = Amount spiked into sample (calculated based on assigned concentration of spiking stock and volume spiked into sample)

Note: The unspiked sample may read 0 pg/mL if the value falls below the detectable range of the standard curve. R&D Systems recommends an acceptable recovery range of 80-120%.
Control Spike should have a recovery value within 80-120%. If not, this indicates there was a problem with preparing the control spike.

2. Linearity

Use the spiked sample value as the Expected Value if testing linearity of the spiked sample.
Use the neat sample value as the Expected Value if testing linearity of the unspiked sample.

% Recovery (1:2) = Observed value (pg/mL) of 1:2 dilution x 100
                   Expected value (pg/mL) divided by 2

% Recovery (1:4) = Observed value (pg/mL) of 1:4 dilution x 100
                   Expected value (pg/ml) divided by 4

% Recovery (1:8) = Observed value (pg/mL) of 1:8 dilution x 100
                   Expected value (pg/mL) divided by 8

Note: Diluting the Control Spike is a good control for serial dilutions. R&D Systems recommends an acceptable recovery range of 80-120% for the Control Spike. If the Control Spike measures outside an acceptable range, this can indicate a problem in preparing the Control Spike dilutions.

Example Calculations

50,000 pg/mL spiking stock solution; 1000 pg/ml total spiked

Values obtained after calculating ODs against standard curve:

Spiked Sample	1250 pg/ml	Control Spike	1010 pg/ml
1:2 Spiked Sample	613 pg/ml	1:2 Control Spike	499 pg/ml
1:4 Spiked Sample	309 pg/ml	1:4 Control Spike	249 pg/ml
1:8 Spiked Sample	162 pg/ml	1:8 Control Spike	123 pg/ml
Unspiked Sample	206 pg/ml		
1:2 Unspiked Sample	101 pg/ml		
1:4 Unspiked Sample	49 pg/ml		
1:8 Unspiked Sample	23 pg/ml		

1: Recovery
% Recovery for Spiked Sample (undiluted) = 1250 pg/ml – 206 pg/ml x 100 = 104%
                                            1000 pg/ml

2: Linearity

Example calculations are for Natural Linearity (unspiked samples)
1:2 Dilution: 101   x 100 = 98%
                (206/2)

1:4 Dilution: 49   x 100 = 95%
               (206/4)

1:8 Dilution: 23   x 100 = 90%
               (206/8)

In this example, Recovery and Linearity fell within the acceptance range of 80-120%

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Conclusions:

If the spike, recovery, and linear dilution studies are in the acceptance range (80-120% as recommended by R&D Systems), values obtained from experimental samples can be determined with confidence.

If spike, recovery and linearity results do not fall within the acceptance range, additional optimization testing (such as changing the reagent diluent or assaying samples at a different dilution) may be necessary.

Additional Resources:

R&D Systems offers a basic ELISA development troubleshooting guide online:
https://web.archive.org/web/20130120084856/http:/www.rndsystems.com/resources/images/5660.pdf
ELISA Development doc:
https://resources.rndsystems.com/images/site/edbapril025670.pdf
ELISA Troubleshooting Guide:
https://www.rndsystems.com/resources/troubleshooting-guide-elisa-development

For more information on ELISA development, please consult a reference guide such as
The Immunoassay Handbook, edited by David Wild, Nature Publishing Group, copyright 2001