Linearity and spike-and-recovery experiments

For quantitation of an analyte in your samples, it is necessary to create a standard curve of your analyte in a diluent that matches your samples. In order to assess whether a proposed diluent is suitable for your sample type, a linearity experiment and a spike-and-recovery experiment should be performed. If you are testing more than one proposed diluent, repeat the experiment(s) as many times as needed for each proposed diluent. A suitable diluent will show good linearity and good percent recovery.

Linearity experiment

1. Spike one of your experimental samples with a high concentration (e.g., 3 ng/mL) of your standard analyte.
2. Perform a 2-fold serial dilution of this spike-in using your proposed diluent to dilute the spike-in. You will want at least five dilutions.

Table 1. Preparation of spike-in dilution series.

Tube	Volume of standard analyte	Volume of diluent to add to sample	Concentration Analyte in tube*	Dilution
1	60 µL of prepared high-concentration spike-in (3 ng/mL)	0	3 ng/mL	1
2	30 µL of prepared high-concentration spike-in (3 ng/mL)	30 µL proposed diluent being tested	1.5 ng/mL	0.5
3	30 µL of tube 2	30 µL proposed diluent being tested	0.75 ng/mL	0.25
4	30 µL of tube 3	30 µL proposed diluent being tested	375 pg/mL	0.125
5	30 µL of tube 4	30 µL proposed diluent being tested	187.5 pg/mL	0.0625
6	30 µL of tube 5	30 µL proposed diluent being tested	93.75 pg/mL	0.03125

*Disregards existing level of analyte in the sample used to create your spike-in, as this is expected to be low in comparison with the high, spiked concentration.

3. In a separate set of tubes, create a standard curve of your standard analyte in the proposed diluent. If you are using an AlphaLISA immunoassay kit, refer to the standard curve concentrations provided in the protocol/tech data sheet. If you are designing your own AlphaLISA immunoassay, you can use concentrations in the range assessed from experiments run in AlphaLISA Immunoassay Buffer. An example is shown below, for the AlphaLISA TNFα kit standard curve.

Table 2. Example for preparation of a TNFα standard curve.

Tube	**Vol. of TNFα (µL)**	**Vol. of diluent (µL) ***	**[TNFα] in standard curve**	
			(g/mL in 5 µL)	**(pg/mL in 5 µL)**
A	100 µL human TNFα standard (100 ng/mL)		1E-07	100 000
B	60 µL of tube A	140	3E-08	30 000
C	60 µL of tube B	120	1E-08	10 000
D	60 µL of tube C	140	3E-09	3 000
E	60 µL of tube D	120	1E-09	1 000
F	60 µL of tube E	140	3E-10	300
G	60 µL of tube F	120	1E-10	100
H	60 µL of tube G	140	3E-11	30
I	60 µL of tube H	120	1E-11	10
J	60 µL of tube I	140	3E-12	3
K	60 µL of tube J	120	1E-12	1
L	60 µL of tube K	140	3E-13	0.3
M ** (background)	0	100	0	0
N ** (background)	0	100	0	0
O ** (background)	0	100	0	0
P ** (background)	0	100	0	0

4. Run your AlphaLISA experimental protocol with your standards, spike-in, and spike-in dilutions (in triplicate). Fit your standard data to a curve and interpolate the concentration of your spike-in and spike-in dilutions.

5. To assess linearity, plot the interpolated concentrations of your spike-in and spike-in dilutions versus the "Dilution" listed in Table 1 (1, 0.5, 0.25, 0.125, 0.0625, 0.03125). Perform a linear regression and assess linearity by correlation coefficient.

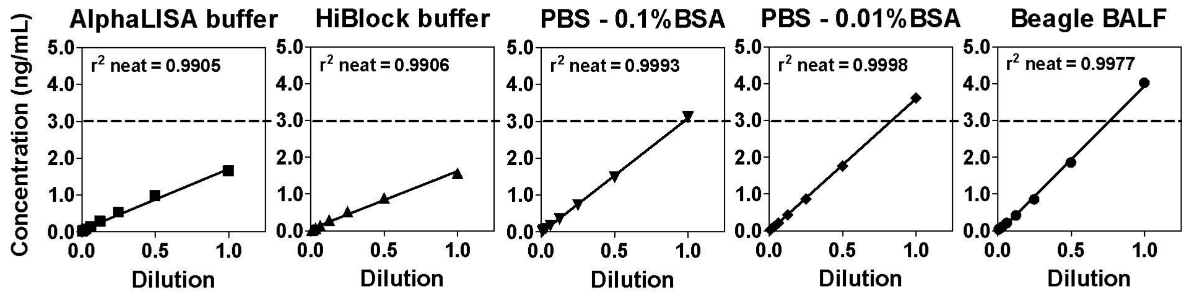

Figure 1. Linearity experiments for an AlphaLISA immunoassay to measure the amount of an analyte in bronchial lavage fluid (BALF) samples. Five linearity experiments were performed to assess the validity of using AlphaLISA Immunoassay Buffer, HiBlock Buffer, PBS + 0.1% BSA, PBS + 0.01% BSA, or Beagle BALF as a diluent for the standard curve. From these experiments, PBS + 0.1% BSA and Beagle BALF were chosen to be further assessed in spike-and-recovery experiments.

If good linearity (>0.995) is not seen with any of the proposed diluents, you may need to dilute your experimental samples 2-fold in order to decrease any interference from the sample matrix. You would adjust your calculated final sample concentrations by multiplying by the dilution factor, 2. The standard curve would be tested in the same diluent used for sample dilution. For more information, refer to the poster "Low volume, highly sensitive immunoassays for detecting cytokines in animal fluids".

Spike-and-recovery experiments

1. In one set of tubes, spike one of your experimental samples with a low, a medium, and a high concentration of your analyte (include one tube that contains sample only – no spike-in). You should choose the low, medium, and high concentrations of analyte based on the dynamic range of your assay (refer to your previous standard curve data).
2. In a second set of tubes, take your proposed diluent (from linearity experiment) and spike with the same concentrations of analyte (include one tube that contains diluent only - no spiked analyte).
3. In a third set of tubes, create a standard curve of your standard analyte in the proposed diluent (refer to step 3 in Linearity Experiments, above).
4. Run your AlphaLISA experimental protocol with your standards and spike-ins. Fit your standard data to a curve and interpolate the concentration of your spike-ins.
5. Compare the interpolated concentrations of your sample spike-ins and diluent spike-ins. You will need to correct the interpolated concentrations of your spiked sample by subtracting the amount of analyte measured from your "No spike" sample from the low, medium, and high spike-in samples. Percent recovery is calculated using the following equation:

$$\text{Percent recovery} = \frac{\text{Calculated concentration of analyte in spiked sample}}{\text{Calculated concentration of spiked diluent}} \times 100$$

Table 3. Percent recovery data for an AlphaLISA immunoassay to measure TNFα in mouse bronchial lavage fluid (BALF) samples. PBS + 0.1% BSA was tested as a diluent for the standard curve. *Concentration for 10-3000 pg/mL spikes is equal to the measured concentration minus the no spike value (in this case, 17.1 pg/mL). Excellent recovery was achieved for all four spikes tested.

	Diluent: PBS + 0.1% BSA		
	Spiked diluent	**Spiked sample (mouse BALF)**	
Spike (pg/mL)	Concentration (pg/mL)	Concentration (pg/mL)*	Recovery (%)
No spike	0	17.1	n/a
10	12	9.2	76
30	35.2	32.3	92
300	300.2	292.6	97
3000	3141.2	2952.1	94