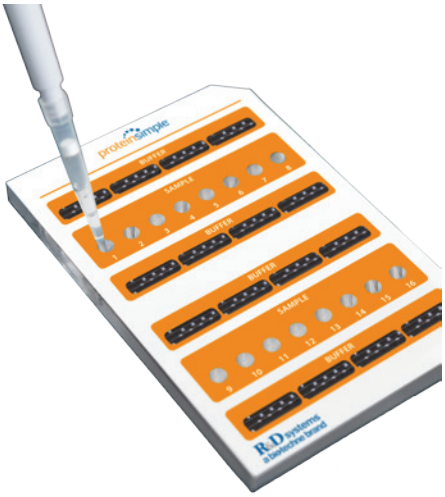
Quick and Simple Validation of Simple Plex Assays

Introduction

Ella's Simple Plex assays give you quantitative answers specific to your favorite proteins in an hour, and only need five minutes of hands-on prep time to boot. That's why scientists use Simple Plex assays in many areas to help indicate disease progression and/or patient response to therapy — from early research and discovery to clinical trials.

Every Simple Plex assay goes through a pretty rigorous validation process at ProteinSimple before it even gets to you. But we also know how important it is to have total confidence in your results. And getting there typically means running an initial internal evaluation to confirm and validate assay specifications before running your own samples. We'll walk you through how to do this evaluation for Simple Plex assays in this application note.



Some Common Terms and Acronyms

Before we get started, here are a few phrases and abbreviations you'll find in this application note.

LLOQ — Lower limit of quantification

ULOQ — Upper limit of quantification

Quantifiable range — The LLOQ to ULOQ range. This is the range where Simple Plex assays accurately determine sample concentration.

Top standard — The combination of four individual proteins at a set concentration. This is the top concentration of the calibration curve which can be used to spike sample with known amounts of protein.

MRD — Minimum required dilution. Check the SAMPLE PREPARATION section of your Assay Specification sheet to get this number.

Linearity (parallelism) — Analysis of serially diluted samples in order to show the proportionality of protein in sample (matrix) to protein in diluent.

Selectivity (spike and recovery) — The accuracy at which spiked protein into a sample or sample matrix can be recovered.

ASSAY PARAMETER	EXPERIMENT DESCRIPTION	MEASURES
Quantitative range	12-point calibration curve	LLOQ and ULOQ
Intra-assay precision	Replicates within a cartridge	%CV (<10%)
Inter-assay precision	Replicates between cartridges	%CV (<15%)
Accuracy (linearity)	Serially diluted sample	% Recovery (75–125%)
Accuracy (selectivity)	Spike and recovery of individual samples	% Recovery (75–125%)

TABLE 1. Parameters to define when evaluating a Simple Plex assay.

Validating Your Assay

The criteria for validating a Simple Plex assay aren't all that different from how you would validate any other immunoassay method. Common experiments to define assay parameters include running calibration curves, characterizing quality controls, confirming linearity, and assessing selectivity **(Table 1)**.

Preparing Simple Plex Protein Standards

Our individual lyophilized Simple Plex proteins can be used as quality controls (QCs) and for evaluation experiments. But you'll need to prep the proteins differently depending on how you ultimately plan to use them. In general, be sure to reconstitute each protein in the sample diluent indicated on the Control Insert, and thoroughly mix for approximately 15 minutes before using.

If you're planning on using the protein for:

Quality Controls (QCs) — Follow the instructions on the Control Insert to prepare your low and high QC control. Just be sure to freeze the aliquots for at least 24 hours at -80 °C before using for stable storage. You can then thaw aliquots as needed.

Evaluation Experiments — Reconstitute the lyophilized proteins that match the four proteins you'll be analyzing with your cartridge. Then, create the top standard by adding 100 µL of each of the four reconstituted proteins to a tube or vial containing 600 µL of sample diluent **(Figure 1)**. The top standards should be made fresh daily and should never be frozen since freeze/thawing will greatly impact the accuracy of your results.

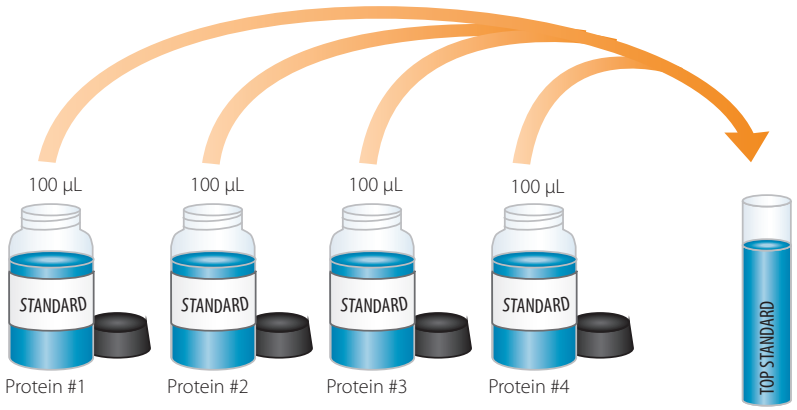


FIGURE 1. Preparing the top standard after reconstituting the Simple Plex proteins.

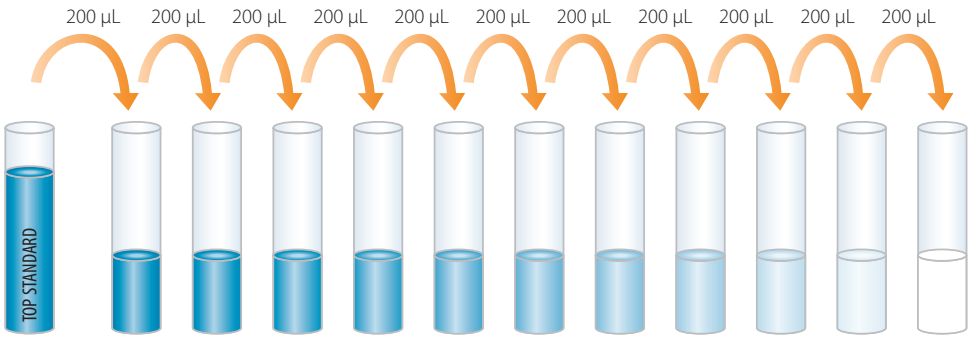


FIGURE 2. Serial dilution of the top standard for a 12-point calibration curve. Pipette 300 µL of sample diluent to each tube/vial.

Determining the Quantitative Range of Your Assay

You'll need to run a 12-point calibration curve to determine the LLOQ and ULOQ of your assay. Start by serially diluting the top standard 2.5-fold by adding 200 µL of top standard to 300 µL of sample diluent **(Figure 2)**. Repeat this another 10 times, and use sample diluent as your zero or blank.

You should run the calibration curve on a minimum of four cartridges to define the quantifiable range of your assay. Just be sure to use your calibration curve within one hour of creating it for best results. Your percent recovery can be calculated as: 100 x (measured concentration/known concentration). Determine the LLOQ and ULOQ based on the criteria below:

LLOQ — The first standard concentration where the %CV is ≤20% and recovery is between 80–120%

ULOQ — The last standard concentration before the %CV is ≥20% and recovery is between 80–120%

To make life simpler, Simple Plex Runner software creates a calibration curve for you. In the Inlet Assignments, select the sample inlets that correspond to your standard curve, then select **Standard** in the Sample Types pull-down menu. Name the standard "standard curve" and then enter the dilution information for your curve.

Click the **Setup Std. Curve** button to automatically generate concentrations for your curve **(Figure 3)**. Deselect **Identical Analytes High Conc** so you can enter the top standard unique concentrations for your four proteins and enter **2.5** for the dilution factor. Then, just check the **With a zero** or **High to Low** boxes if you loaded your curve high to low or if you're including a zero and you're done! If you need a bit more detail on setting up a calibration curve, check out the Ella User Guide.

Add your samples and buffers to the Simple Plex Cartridge and start your run — prep only takes five minutes. The run will be done in about an hour, and once it is, you can build your calibration curve in Simple Plex Explorer software. In the File menu, click **Load Kits** and select all the kits you want to make a comprehensive curve from. Then select **Build a Curve** in the Tools menu. On a per protein basis, choose the protein from the drop-down menu, click **Standard** under Sample Type, then right-click on RFU Results from Kits to select all. All the data from the kits you selected display in the graph on the right **(Figure 4)**.

You can also fine-tune your curve fit. In the Curve window, scroll over the data point to see its x, y data. Points from the curve can be removed by deselecting them from RFU Results from Kits. You can also sort the data in the RFU Results from Kits window by clicking the column headers.

When you're done, save the curve. If you need to export the curve, just select **Export to Clipboard.**

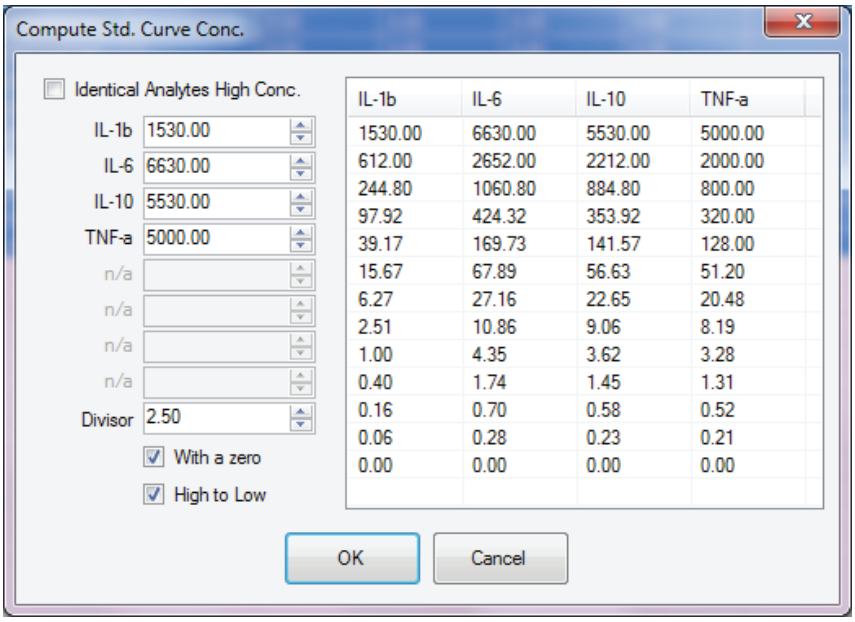


FIGURE 3. Using the Setup Std. Curve function to automatically calculate standard curve concentrations in Simple Plex Runner software.

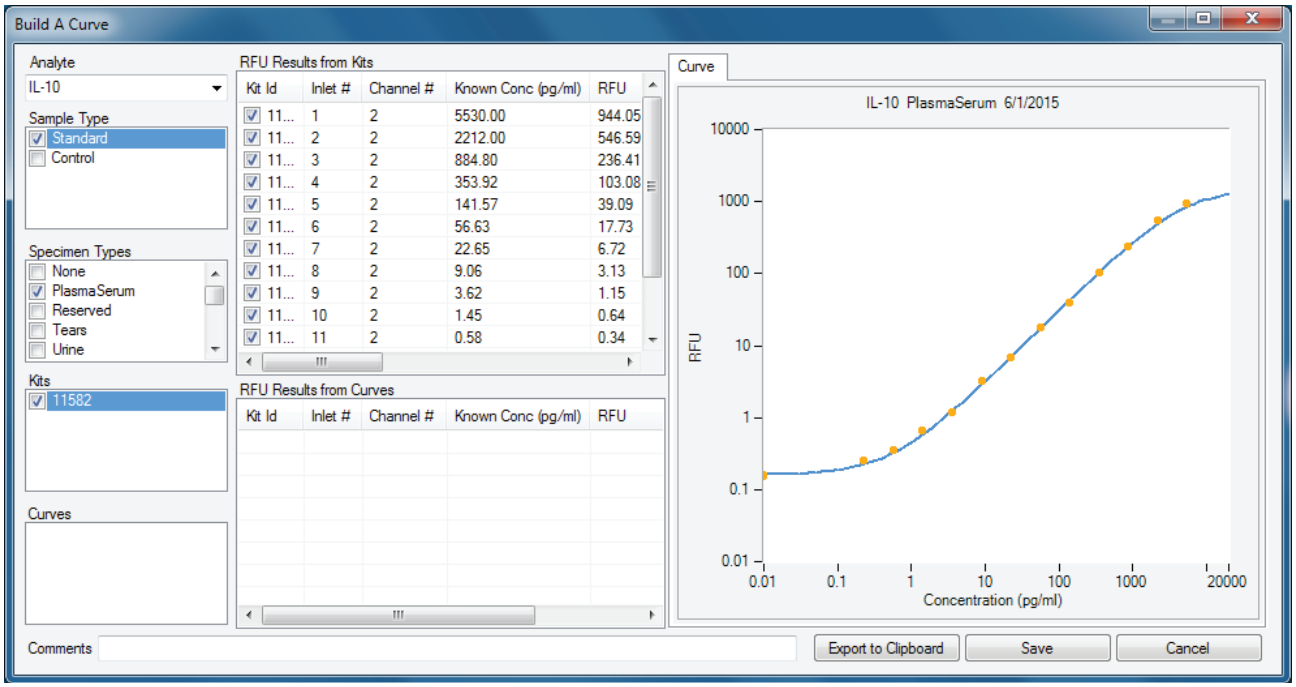


FIGURE 4. Building a calibration curve in Simple Plex Explorer software.

Determining Assay Precision

You can also characterize quality controls to assess assay precision. If you decide to go this way, you'll want to determine both the intra-assay precision as well as the inter-assay precision.

To determine the intra-assay precision, calculate the %CV for low and high QC samples over a minimum of eight samples in a single cartridge. Look for %CVs that are <10%. For inter-assay precision, calculate the %CV for low and high QC samples over a minimum of four cartridges, %CVs here should be <15%.

Determining Assay Accuracy

The next step is to assess linearity to determine your assay's accuracy. If the endogenous amount of protein is well above the LLOQ, just dilute your sample according to the MRD indicated on the Assay Specification sheet. Then do 2-fold serial dilutions of your sample in sample diluent. The number of dilutions you should prepare in addition to the MRD will depend on the amount of endogenous protein present in your sample. If endogenous levels are near or below the LLOQ of the assay, use the top standard to spike the sample, but take care not to spike more than 5% of the final sample volume. Then perform the same dilutions mentioned earlier **(Figure 5)**.

To assess the linearity accuracy, calculate the percent recovery as: calculated concentration/expected concentration. In this case, the expected concentration is the measured concentration at MRD **(Table 2)**. And there's no need to calculate recoveries for samples that measured outside the quantifiable range. Your assay is linear when recovery at all dilutions is between 75–125%.

If you want to refit your sample data to your own standard curve with Simple Plex Explorer, select **Load Kits** and **Load Curves** in the File menu. Then, in the Tools menu select **Apply Curves to Kits** and select your kits and curves of interest. Once you click **Apply Selected Curves to Selected Kits**, your data will automatically refit **(Figure 6)**. When that's done, save your changes by clicking **Save**, or **Save As** if you want to change the file name. You can always revert any kit back to the data generated using the factory calibration curve by clicking **Apply Factory Curves**.

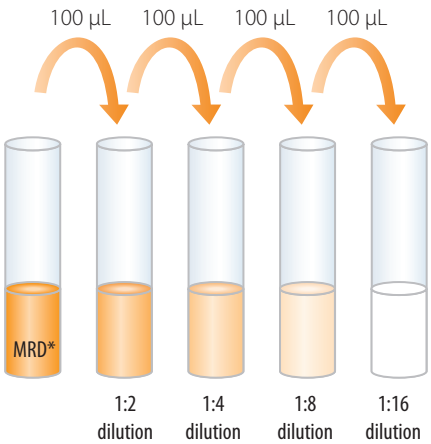


FIGURE 5. Dilute your sample according to the MRD in the Assay Specification Sheet, followed by serial 2-fold dilutions with equal part sample to sample diluent (100 µL each).

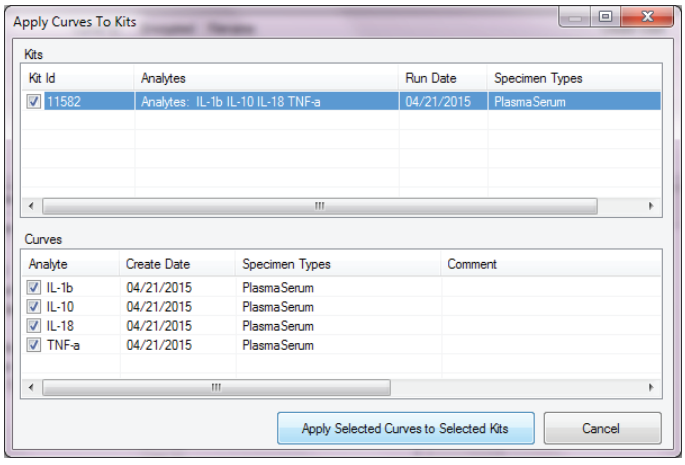


FIGURE 6. Refitting your sample data to your own calibration curve.

CONDITION	IL-1β		IL-6		IL-10		TNF-α	
	CONC (pg/mL)	RECOVERY	CONC (pg/mL)	RECOVERY	CONC (pg/mL)	RECOVERY	CONC (pg/mL)	RECOVERY
MRD	669.78		1031.2		676.04		1418.72	
1:2 Dilution	717.64	107%	1133.68	110%	709.92	105%	1575.12	111%
1:4 Dilution	738	110%	1218.72	118%	754.4	112%	1600.4	113%
1:8 Dilution	725.92	108%	1215.36	118%	775.04	115%	1601.44	113%
1:16 Dilution	714.56	107%	1209.92	117%	688.64	102%	1511.68	107%

TABLE 2. Spiked linearity for IL-1β, IL-6, IL-10, and TNF-α at four 2-fold dilutions above the MRD for a human serum sample. To make life easier, Simple Plex Explorer software normalizes the concentration based on the sample dilution factor.

SOLUTION	TOP STANDARD CONC	SPIKE MATERIAL	MATRIX MATERIAL	DILUTION
Spiking solution	2X	Protein stock(s)	Sample diluent	1:5
High spike	0.1X	Spiking solution	Sample or sample diluent	1:20
Medium spike	0.025X	High spike	Sample or sample diluent	1:4
Low spike	0.00625X	Medium spike	Sample or sample diluent	1:4
Unspiked	0X	N/A	Sample	N/A

TABLE 3. Create a spiking solution with 1:5 dilutions of the freshly reconstituted lyophilized stock proteins. Dilute spiking solution 1:20 in either sample or sample diluent with subsequent 1:4 serial dilutions.

When Spiked Linearity isn't Enough

When endogenous levels are low and linearity can only be achieved by spiking protein into your sample, you'll also want to assess spike and recovery. To do this, spike known concentrations of protein into your sample(s) using the stock proteins, again taking care that the spiking solution doesn't represent more than 5% of the final volume. From the first spiked sample, perform serial dilutions in sample matrix to assess the accuracy at low, medium and high concentrations, respectively **(Table 3)**. Then perform the same dilution scheme in sample diluent to act as the control spike.

Dilute spiked and unspiked samples in sample diluent according to the MRD listed in the Assay Specification sheet. Load control spikes undiluted and then calculate the % recovery **(Table 4)**.

Calculate percent recovery as:

$$\left(\frac{\textit{(Spiked sample measured concentration - endogenous measured concentration)}}{\textit{Spiked control measured concentration}} \right) \textit{x 100}$$

Recoveries between 75-125% indicate your assay has good accuracy.

See the *Determining Assay Accuracy* section for instructions on how to apply sample data to an internally generated calibration curve.

CONDITION	IL-1β		IL-6		IL-10		TNF-α	
	CONC (pg/mL)	RECOVERY	CONC (pg/mL)	RECOVERY	CONC (pg/mL)	RECOVERY	CONC (pg/mL)	RECOVERY
Control - low spike	30.28	N/A	19.93	N/A	13.80	N/A	57.88	N/A
Control - med spike	96.92	N/A	73.29	N/A	50.63	N/A	178.70	N/A
Control - high spike	228.71	N/A	161.62	N/A	118.80	N/A	437.25	N/A
Endogenous	0.73	N/A	2.83	N/A	0.80	N/A	5.65	N/A
Low spiked sample	34.82	113%	22.66	99%	14.40	99%	62.05	97%
Med spiked sample	106.19	109%	73.75	97%	54.24	106%	181.10	98%
High spiked sample	238.16	104%	145.09	88%	106.67	89%	394.22	89%

TABLE 4. IL-1β, IL-6, IL-10, and TNF-α at three spiked levels for a human serum sample. The % recovery for all three assays was between 75–125% confirming assay accuracy.

Conclusion

ProteinSimple validates every Simple Plex assay before it goes out the door, but when you want to validate results before running your own samples the steps to do that are pretty simple. Most evaluations include running a calibration curve, analyzing replicates within a cartridge and between different cartridges, and spike and recovery experiments. And because Simple Plex assays are done in an hour and only need five minutes of hands-on prep time, the whole process is pretty quick too. Once the evaluation is complete, you'll have total peace of mind in knowing the quantitative range, precision, and accuracy of the data Ella generates for your protein is spot-on!

proteinsimple

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