

The New Gold Standard for Gene Therapy Protein Expression Potency Measurements

Simple Western™: Analytical-Grade Protein Expression Quantification in a Multi-Attribute Platform Approach

Gene Therapy Potency Measurements Are in Need of Potent Analytical Methods

A major challenge preventing transformative [gene therapies](#) from reaching patients is the lack of fit-for-purpose potency assays, which are some of the most important assays in an analytical package but are often the most complex and high-risk.¹⁻⁵ Defined as “the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result,” potency assays are used for process development testing, analytical characterization, QC release testing, stability studies, and comparability studies.² Therefore, potency assays must be quantitative, dose-responsive, and well-controlled. Because of their complex mechanism of action, potency assays require a matrix approach of multiple complementary assays, incorporating infectivity, transgene mRNA and protein expression, and biological activity.

ELISA is a workhorse for protein expression potency analysis,⁶ but limitations include interference by matrix effects, challenging assay development, and overestimation of analytes due to cross-reactivity. Western blot is still used in clinical trials despite poor reproducibility, limited quantitation, large sample volume requirements, and low throughput.⁷ Flow cytometry is limited by background interference,⁸ and the lack of validated antibodies can complicate [GMP](#) requirements. Finally, PCR methods are not fully predictive of protein expression.⁶

As a result, new fit-for-purpose protein expression potency assays are needed, ideally in a platform approach that will enable efficient, simple, and inexpensive upstream and downstream process development.⁹

Get the Flexibility of Western Blots with the Analytical-Grade Quantitation of ELISA

Simple Western™ is a capillary electrophoresis (CE) platform that pairs [molecular weight \(MW\)](#) or [isoelectric point separation](#) with a quantitative immunoassay read-out and represents the next-generation gold standard for quantitative, reproducible, and highly specific analytical-grade protein expression potency assays. By pairing the flexibility and specificity of Western blots with the analytical-grade quantitation of ELISA, Simple Western is ideal for making specific measurements in complex sample types like tissue homogenates and analyzing intracellular protein expression without [matrix effects](#) that can confound ELISA read-outs.

As a platform technology that needs only a single validated antibody and offers rapid 3-hour runtimes, Simple Western lowers the custom assay development hurdles of ELISA and accelerates drug development from pre-clinical phases and scaling to GMP. Plus, Simple Western is a multi-attribute method used in gene therapy development, beyond protein expression potency assays, for quantitative CQA monitoring of [purity](#), [titer](#), [empty/full capsid content](#), and [capsid protein ratio](#).¹⁰⁻¹⁵

In this Application Note, we demonstrate a Simple Western relative potency assay with parallel line analysis (PLA) for AAV-mediated gene delivery in cultured human cells. Specifically, we demonstrate:

- Relative protein expression potency between AAV serotypes with high specificity and <5% CVs
- Absolute protein expression measurements in AAV-transduced cells using a recombinant standard curve
- Detection of residual intracellular capsid proteins in AAV transduced cells as an infectivity surrogate

By measuring GFP normalized to total protein directly in transduced cells, we show that AAV2 is significantly more potent than AAV9 with highly reproducible quantitation that is 10X more sensitive than flow cytometry, with improved specificity and less matrix effect than ELISA.¹⁶

Materials and Methods

Relative Protein Expression Potency Assay

The materials used for the relative potency assay are listed in TABLE 1. Serotypes AAV2 and AAV9 were used to transduce the human liver cancer cells (the HepG2 liver cell line), which has been used for *in vivo* potency assays for AAV gene therapy.¹⁷ These vectors were engineered to deliver and express GFP driven by the CMV promoter, an established reporter of AAV transduction in HepG2 cells,¹⁷ by random genomic integration with no homology sequences flanking the CMV-GFP transgene.

AAV Transduction of Human Liver Cells

HepG2 cells were transduced in duplicate with AAV2 or AAV9 vectors with CMV-GFP genetic payloads in a 7-point titration of 1×10^6 , 2×10^5 , 4×10^4 , 8×10^3 , 1.6×10^3 , 3.2×10^2 , and 6.4×10^1 vector genomes per cell (vg/cell). Empty AAV2 and AAV9 were included as negative controls. At 48 hours post-transduction, 6×10^6 cells were collected from each vial and control treatment and split into 3 Eppendorf tubes (2×10^6 cells per tube). Cells were spun at $1000 \times g$ for 2 minutes, the supernatant was removed, and the cells were washed with 1 mL of PBS. Cells were spun again at $1000 \times g$ for 2 minutes and the supernatant was removed, and the tubes were placed on dry ice for 10 minutes to flash freeze the cells and stored at -80°C .

Simple Western Analysis

All samples were prepared as described in the 12-230 kDa Separation Module product insert. The anti-GFP antibody was diluted to $20 \mu\text{g/mL}$ in Milk-Free Antibody Diluent. All samples were analyzed on Simple Western's

JessTM instrument with default settings. Relative potency was determined by three Simple Western runs with individually prepared samples at a concentration of 0.1 mg/mL . For this study, AAV9 was selected as the reference material and AAV2 as the test material. GFP and total protein were detected in Probe 1 and Probe 2 of the RePlexTM assay, respectively.

Parallel Line Analysis (PLA)

Raw data and data normalized to total protein were used to determine relative potency using the PLA method. See the Appendix for more information on the PLA method using GraphPad Prism.

Absolute Protein Expression Measurements

To quantify absolute GFP protein expression levels, a 3X serial dilution series of recombinant GFP (rGFP) was prepared from 5 ng/mL to 0.06 ng/mL and analyzed in the same Simple Western run as the AAV2 or AAV9-transduced cells. The peak areas resulting from rGFP analysis were plotted by rGFP concentration, and a log-log regression with $1/y^2$ weighting was applied to create a standard calibration curve that was used to quantify absolute GFP expression in transduced cells. All samples were analyzed in duplicate.

Detection of Intracellular AAV VP1/VP2/VP3

The materials used for intracellular AAV detection are listed in TABLE 2. All samples were prepared as described in the 66-440 kDa Separation Module product insert, except the sample denaturation was performed at 70°C for 10 minutes. The anti-VP1/VP2/VP3 antibody was diluted to $20 \mu\text{g/mL}$ in Antibody Diluent 2 and the anti-mouse-HRP secondary antibody was diluted to 1X in Antibody Diluent 2. All samples were analyzed in duplicate on Simple Western's Jess instrument with default settings, except the sample load time was adjusted to 6 seconds.

TABLE 1. Materials used for relative potency analysis

Item	Vendor	Part Number
12-230 kDa Separation Module		SM-W004
EZ Standard Pack 2		PS-ST02EZ-8
Anti-Goat Detection Module		DM-006
Total Protein Detection Module	Bio-Techne	DM-TP01
RePlex TM Module		RP-001
Recombinant Jellyfish GFP Protein		NBC1-22949
Anti-GFP Antibody		AF4240
AAV2/9 with CMV-GFP and empty	Virovek	N/A

TABLE 2. Materials used for VP1/2/3 detection

Item	Vendor	Part Number
66-440 kDa Separation Module		SM-W008
Goat-Anti-Mouse-HRP Antibody		040-655
Streptavidin-HRP	Bio-Techne	042-414
Luminol-S		043-311
Peroxide		043-379
Antibody Diluent 2		042-203
Anti-AAV VP1/VP2/VP3 Antibody	PROGEN	690058S
AAV2 vector stock	Virovek	N/A

Results of the Simple Western Relative Protein Expression Potency Assay

Finding the Log-Log Linear Range of the Assay

Initial sample evaluation was performed to determine the concentration of the reference and test sample with a log-log linear range of detection. In this study, AAV2 was considered the test sample and AAV9 as the reference.

Whole-cell lysates of HepG2 cells transduced with a 5X 7-point serial dilution series from 1×10^6 to 6.4×10^1 AAV2 or AAV9 were analyzed at concentrations of 1, 0.1, and 0.01 mg/mL. When these samples were analyzed on Jess using an anti-GFP antibody, the results showed that AAV2-transduced GFP expression reached saturation at 1 mg/mL (FIGURE 1, top panel), and the GFP signal was very low in AAV9-transduced cells at lower dosages (FIGURE 1, middle panel). At a concentration of 0.1 mg/mL, GFP expression was linear from 6.4×10^1 to 8×10^3 vg/cell of AAV2 and from 1.6×10^3 to 1×10^6 vg/cell of AAV9 (FIGURE 1, bottom panel).

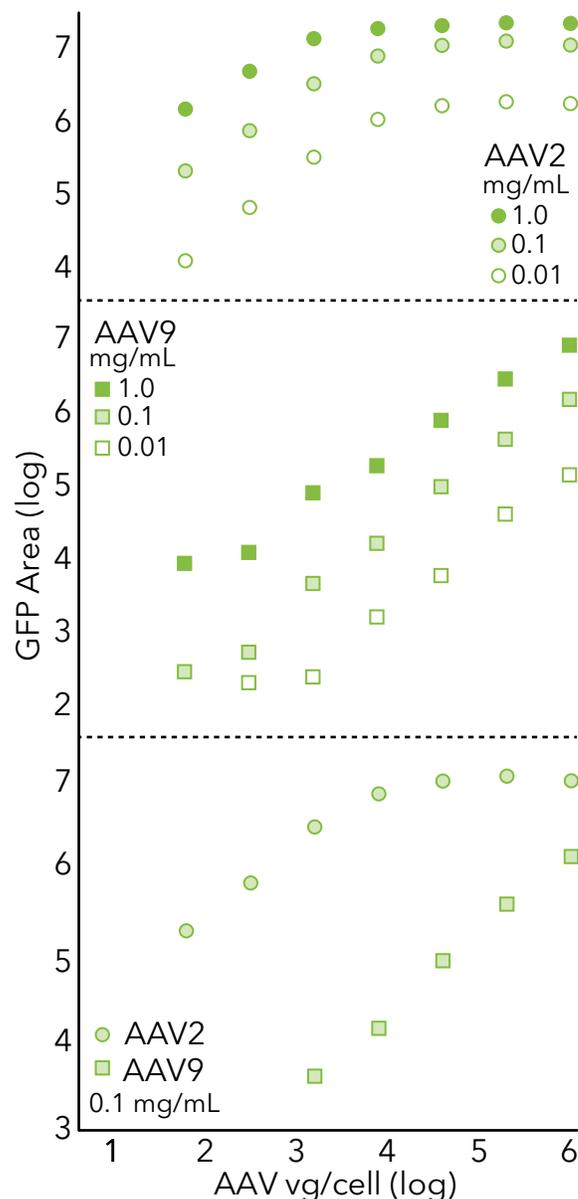
Based on these results, relative potency was determined at a final whole-cell lysate concentration of 0.1 mg/mL containing cells transduced with AAV2 or AAV9 with 5-point viral titrations (vg/cell) of 6.4×10^1 to 4×10^4 and 1.6×10^3 to 1×10^6 for AAV2 and AAV9, respectively.

Measuring Relative Potency of AAV2 to AAV9

Using the sample concentration of 0.1 mg/mL established above, Simple Western analysis of transduced liver cells showed that the maximum GFP expression for AAV2 occurred with titers $>4 \times 10^4$ vg/cell, and the maximum AAV9 GFP expression occurred with titers $>1 \times 10^6$ vg/cell (FIGURE 2A). These results were consistent with flow cytometry analysis of cells from the same sample timepoint, with AAV2 transduction resulting in significantly higher GFP expression than AAV9 (FIGURE 2B). However, Simple Western was more sensitive than flow cytometry because GFP was detectable in cells transduced with AAV9 titers as low as 1.6×10^3 vg/cell, while flow cytometry required AAV9 titers of at least 4.0×10^4 vg/cell. Thus, detection by Simple Western was 10-fold more sensitive than flow cytometry under the conditions tested here.

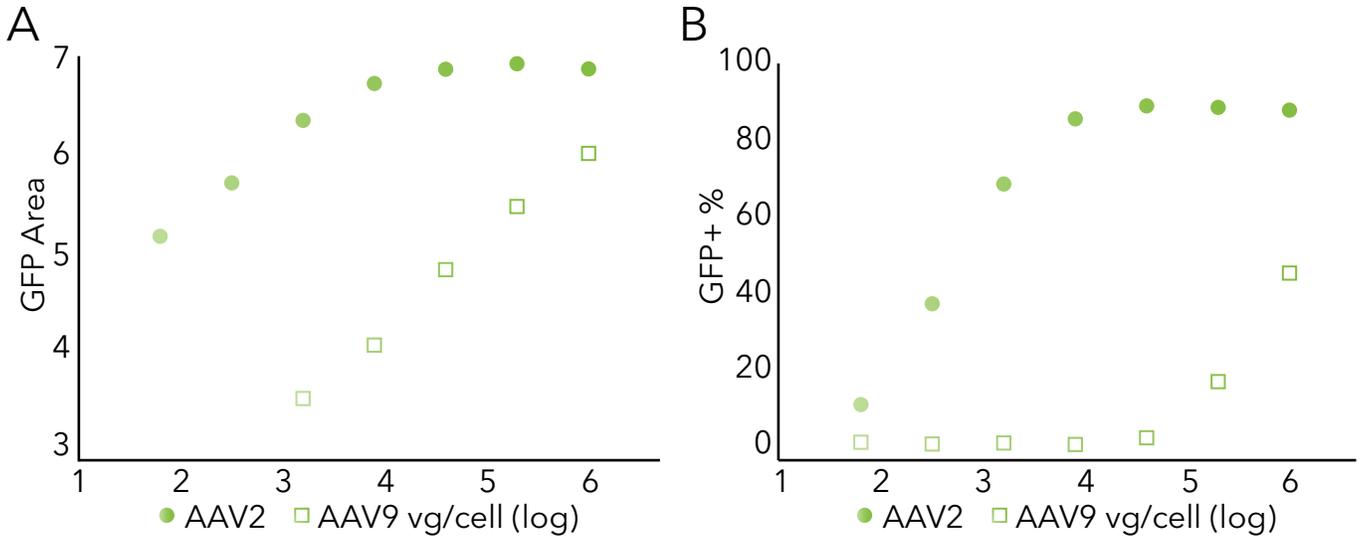
We used the RePlex feature on Jess to perform sequential immunoassay and total protein analysis using an anti-GFP antibody in Probe 1 (FIGURE 3, green overlays) and total protein detection in Probe 2 of RePlex (FIGURE 3, blue overlays). This method was repeated 3 times with independently-prepared samples to calculate relative potency using the PLA method described below.

FIGURE 1. Finding the log-log linear range of the Simple Western relative potency assay.



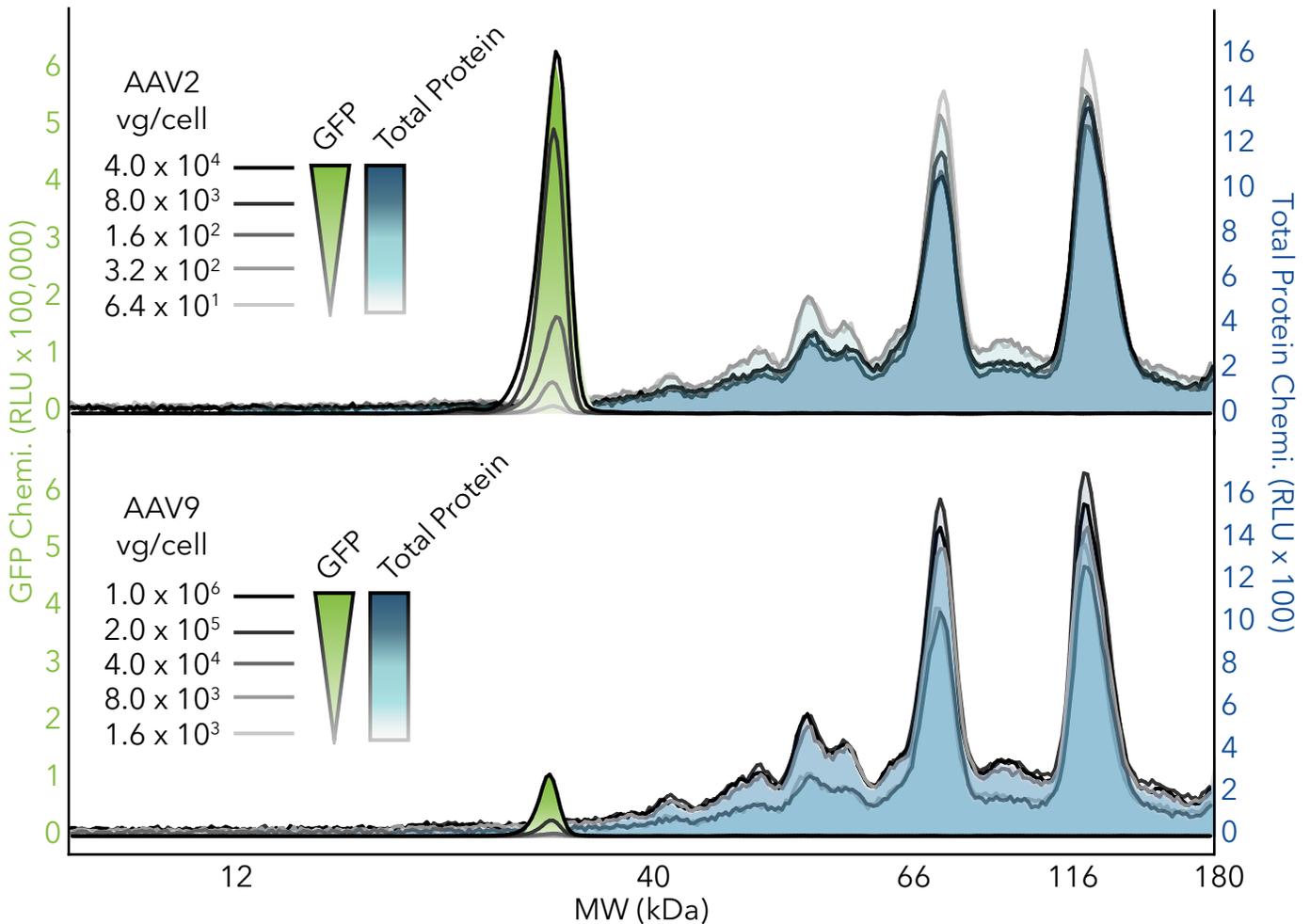
Whole-cell lysates of HepG2 cells transduced with AAV2 (circles) and AAV9 (squares) were analyzed at final concentrations of 1 mg/mL (filled shapes), 0.1 mg/mL (shaded shapes), and 0.01 mg/mL (open shapes). Peak areas of transgene GFP expression were measured using an anti-GFP antibody.

FIGURE 2. (A) Simple Western and (B) flow cytometry analysis of AAV-mediated GFP transgene expression



(A) GFP expression was measured by Simple Western in whole-cell lysates (0.1 mg/mL) of cells that were flash-frozen at 48 hours post-transduction with AAV2 (filled circles) and AAV9 (outlined squares). (B) Percent GFP-positive cells (GFP+ %) were measured by flow cytometry from the same 48-hour sampling time.

FIGURE 3. Simple Western analysis of AAV-mediated GFP transgene expression with total protein detection



The overlaid electropherograms resulting from Simple Western analysis of whole-cell lysates of liver cells transduced with serial dilutions series of AAV2 (top panel) and AAV9 (bottom panel) to deliver CMV-GFP genetic payload. Samples were probed with an anti-GFP antibody in Probe 1 of RePlex (green overlays) and total protein detection was performed in Probe 2 of RePlex (blue overlays).

Parallel Line Analysis (PLA)

The relative potency assay was calculated from Simple Western measurements described above (FIGURE 3) using the PLA method (FIGURE 4). A minimum of 3 points per sample is required to determine parallelism. The top point for the AAV2 series fell above the upper range limit of our assay based on initial assay development and therefore had to be removed from the analysis. Comparing the accepted data points, the slopes between the two linear regressions were not significantly different, enabling parallel line analysis of relative potency (FIGURE 4). See the Appendix for more information on how to perform the PLA method with GraphPad Prism.

The relative potency of AAV2 to AAV9 was calculated for each run, and the mean and inter-assay coefficients of variability (CV) were calculated across all three runs (TABLE 3). Consistent with reported results,¹⁷ Simple Western established AAV2 as a significantly more potent serotype than AAV9 for transduction of HepG2 cells, with

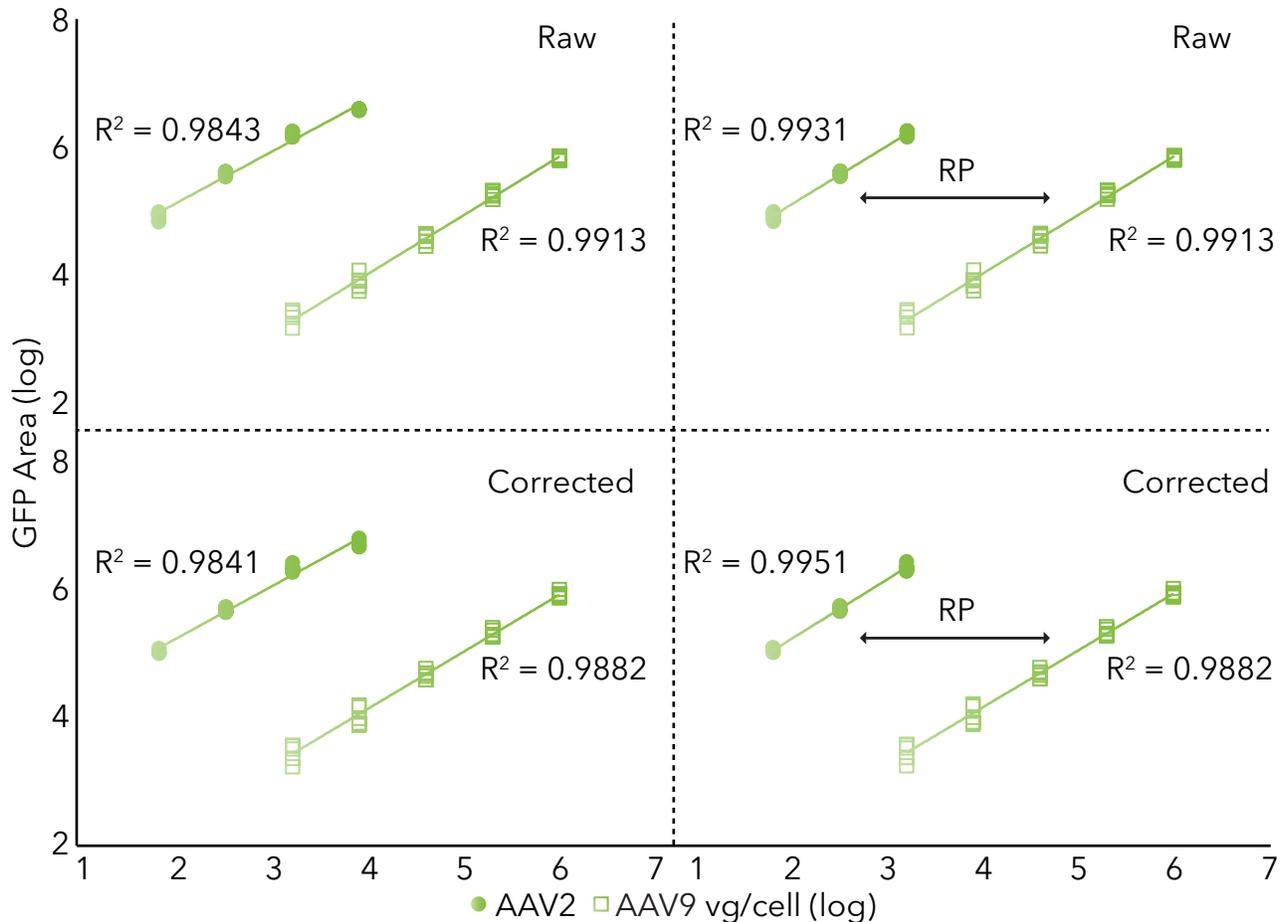
a mean relative potency of 1452 and 1651 for raw and total protein-normalized values, respectively (TABLE 3). When normalization of protein expression data is required, total protein is more reliable than using a housekeeping protein as the denominator.¹⁸ Furthermore, the quantification of relative potency by Simple Western was highly reproducible, with inter-assay CVs of 2.0% and 4.8% for raw and normalized values, respectively (TABLE 3).

TABLE 3. AAV2 potency relative to AAV9

	Raw	Normalized
Run 1	1423	1597
Run 2	1451	1614
Run 3	1481	1743
Mean	1452	1651
%CV	2.0%	4.8%

Relative potency was determined from three Simple Western runs with independently-prepared samples. Raw and total protein-normalized values were calculated by the PLA method using GraphPad Prism (see Appendix).

FIGURE 4. Simple Western parallel line analysis for calculating relative protein expression potency



GFP peak areas were plotted by the log AAV titer for raw values (top) and total protein-normalized values (bottom). When the highest two points of the AAV2 titration series were removed, the resulting slopes were not significantly different according to GraphPad analysis (see Appendix).

Absolute Protein Expression Measurements in AAV-Transduced Cells

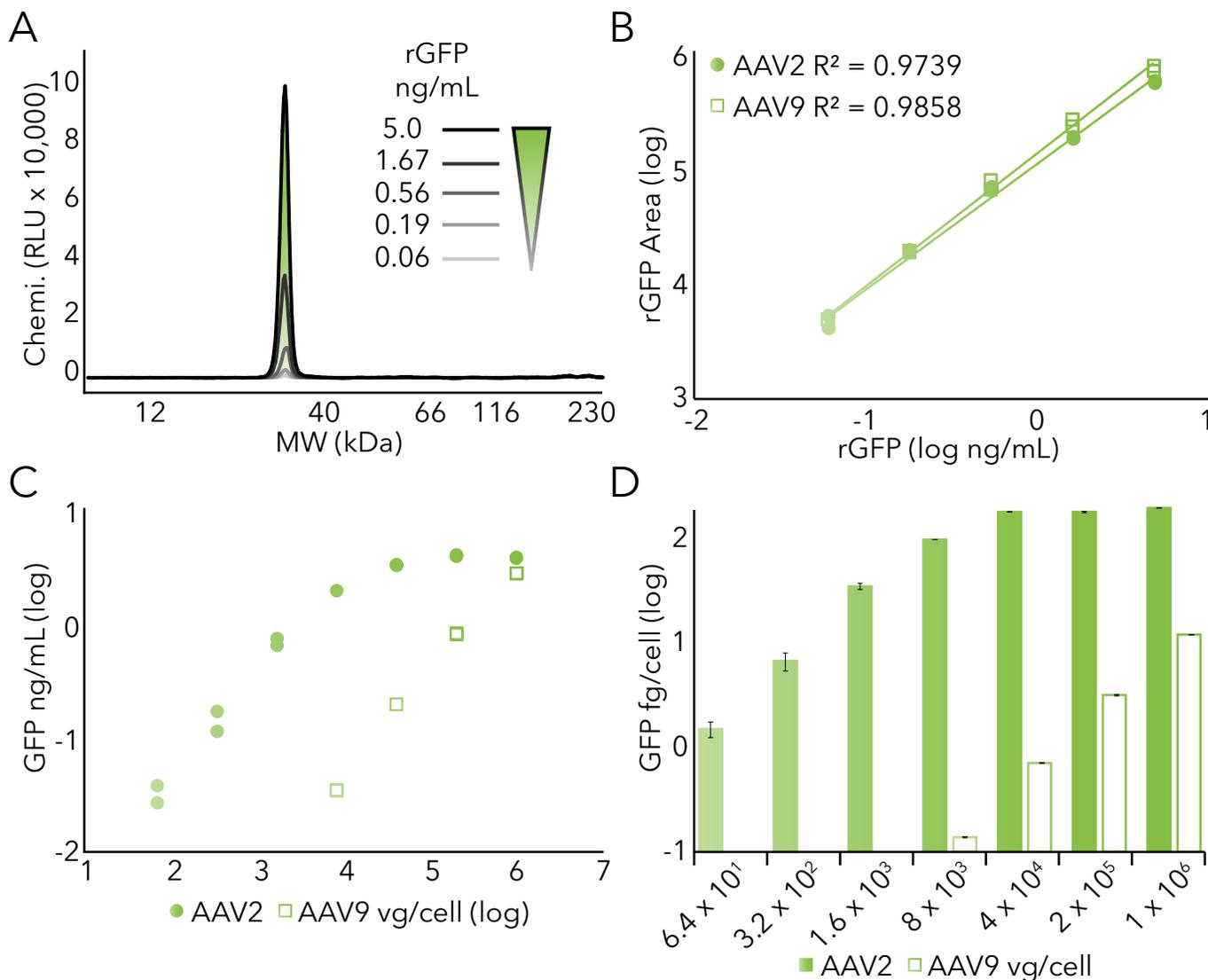
To quantify absolute protein concentrations of transgene GFP expression in AAV-transduced cells, we prepared a serial dilution series of purified recombinant GFP (rGFP) that was analyzed in the same run as the test samples of transduced cells. Log-log regression with $1/y^2$ weighting was applied to the rGFP peak areas plotted by rGFP concentrations to create a standard calibration curve, which was used to quantify absolute GFP concentrations in AAV transduced samples (FIGURE 5B). As expected, significantly higher absolute GFP concentrations were measured with AAV2, as high as 3913 ng/mL (TABLE 4 and FIGURE 5C-D). We also calculated absolute GFP expression per cell, which ranged from 196 fg/cell down to 1 fg/cell at the highest and lowest AAV2 viral loads, respectively (TABLE 4 and FIGURE 5D).

TABLE 4. Absolute GFP expression measurements

AAV Titer	AAV2-Transduced Cells		AAV9-Transduced Cells	
vg/cell	GFP ng/mL	GFP fg/cell	GFP ng/mL	GFP fg/cell
1.0×10^6	3913	196	239	12
2.0×10^5	3579	179	63	3
4.0×10^4	3593	180	14	1
8.0×10^3	1950	97	3	0
1.6×10^3	697	35	ND	
3.2×10^2	134	7	ND	
6.4×10^1	30	1	ND	

GFP expression was not detectable (ND) in cells transduced with AAV9 of titers $\leq 1.6 \times 10^3$ vg/cell.

FIGURE 5. Absolute quantification of GFP expression levels in AAV-transduced liver cells



GFP expression was not detectable in cells transduced with AAV9 of titers $\leq 1.6 \times 10^3$ vg/cell. Error bars represent standard errors of the means.

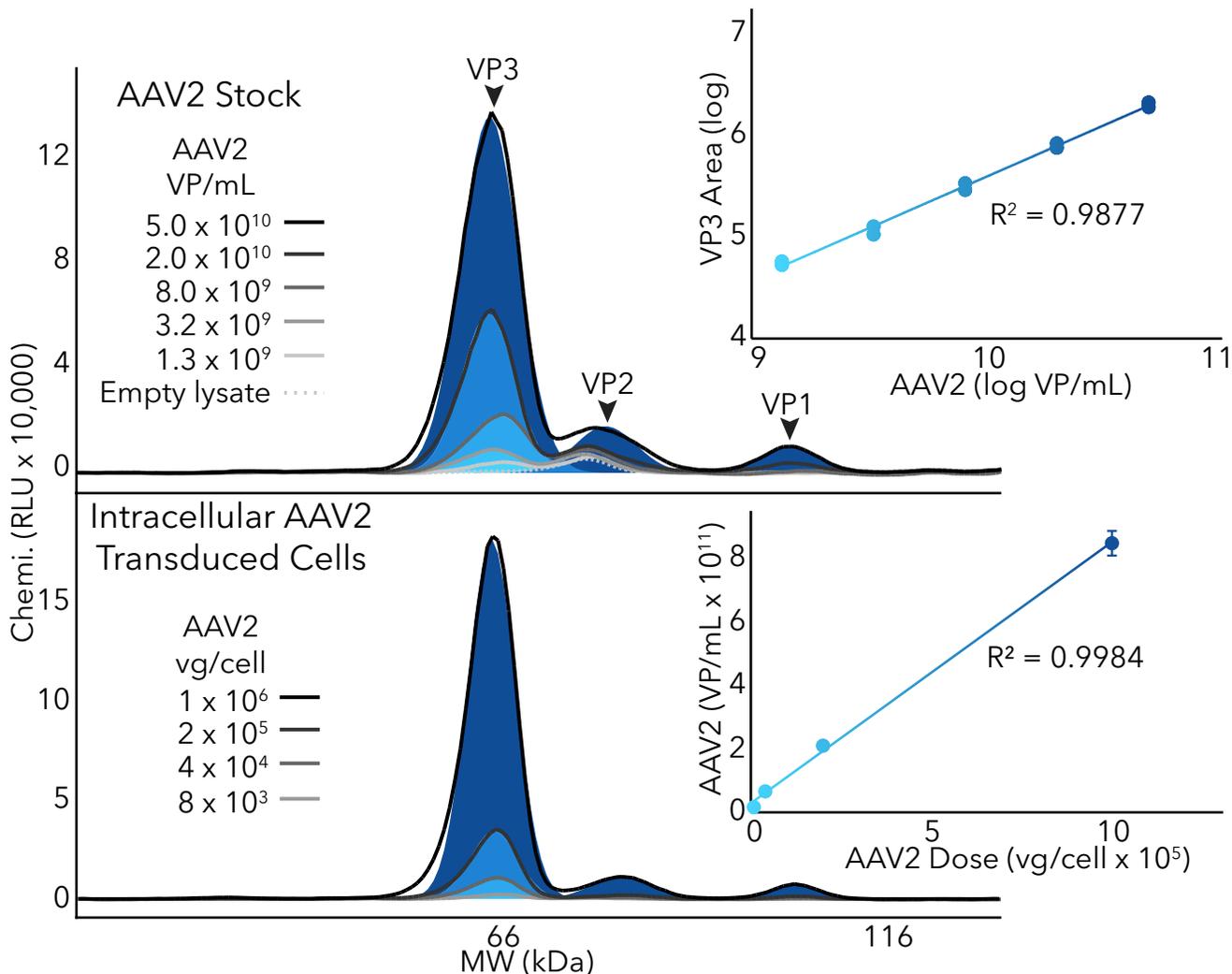
Screening for Intracellular AAV Capsid Proteins in Transduced Cells as an Infectivity Surrogate

We tested if Simple Western could detect the persistence of AAV capsid proteins in cells post-transduction. As a positive control, a serial dilution series of purified AAV2 stock was prepared in a HepG2 lysate background and analyzed using an anti-VP1/2/3 antibody. Clear signals corresponding to VP1/2/3 appeared that decreased with decreasing vector concentration (FIGURE 6, top panel). To create a standard curve, the VP3 peak area from each sample was plotted by AAV2 concentration, and log-log regression with $1/y^2$ weighting from 1.3×10^9 to 5.0×10^{10} VP/mL (FIGURE 6, top panel inset). Next, cells transduced with the 4 highest AAV2 titers (FIGURE 1, 8×10^3 to 1×10^6 vg/cell) were analyzed with the anti-VP1/2/3 antibody, revealing a similar VP1/2/3 separation profile as the AAV2 stock control (FIGURE 6, bottom panel). Finally, we quantified the absolute concentrations of AAV2

in transduced cells using the VP3 peak areas and the standard curve, resulting in AAV2 concentrations of 1.67×10^{10} to 8.41×10^{11} VP/mL (adjusted for dilution) that directly correlated with the viral loads used for transduction (FIGURE 6, bottom panel inset).

TCID₅₀ assays are used to measure infectivity, but they can be unreliable and are not easily amenable to scale. To our knowledge, this is the first time Simple Western was used for absolute quantification of AAV vectors directly in transduced cells as a surrogate for AAV infectivity and a potential rapid, multi-attribute alternative to the TCID₅₀ assays. The persistence of AAV *in vivo* is clinically meaningful as residual AAV capsid proteins may be immunogenic and can impact patient safety following the administration of an AAV gene therapy product.

FIGURE 6. Intracellular detection of VP1/2/3 in transduced liver cells as a surrogate for AAV infectivity



Simple Western analysis of AAV2 vector stock prepared in an untreated HepG2 whole-cell lysate background diluted 1:5 (top panel) and HepG2 whole-cell lysates diluted 1:20 of cells transduced with AAV2 (bottom panel) using an anti-VP1/2/3 antibody. The VP3 peak area of the AAV2 stock was plotted by stock concentration to create a standard curve (top panel inset), and used to quantify AAV2 in transduced cells, which was adjusted by dilution and plotted by dosage (bottom panel inset). Cell transduced with AAV9 did not give a detectable signal using the anti-VP1/2/3 antibody (data not shown).

A Fit-For-Purpose Potency Assay

Here, we established [Simple Western](#) as a specific, highly reproducible, and quantitative tool for AAV relative potency measurements. The relative potency between AAV2 and AAV9 serotypes was determined by measuring GFP transgene expression normalized to total protein, which is detected automatically in the same sample capillary using Simple Western's RePlex feature. Ultimately, Simple Western conclusively demonstrated AAV2 as a significantly more potent serotype than AAV9, which is consistent with reported results.¹⁷ The assay was highly quantitative and reproducible, with a CV <5% across three independently prepared samples and runs.

The results obtained with Simple Western which showed more potent transduction and GFP expression by AAV2 compared to AAV9 were consistent with flow cytometry analysis. However, Simple Western was more than ten times more sensitive than flow cytometry under the conditions tested here. Simple Western has the added advantage of detecting intracellular proteins, which can be challenging by flow cytometry. Furthermore, Simple Western provided absolute protein expression measurements directly in cells using a standard curve.

Simple Western is a fit-for-purpose tool for quantitative gene therapy potency assays to quantify vector-mediated protein expression because Simple Western can work with a variety of complex sample matrices with minimal matrix effects.¹⁶ Compared to traditional methods like ELISA and Western blot, Simple Western requires much smaller protein loads, preserving precious tissue samples. Simple Western also provides increased automation, which allows for better standardization across multiple sites, as well as higher throughput, which will allow for increased biological and technical replicates for increased accuracy.

A Multi-Attribute Platform Approach for Viral Vector Characterization and Manufacturing

As an open immunoassay platform, any antibody may be used for detection with the advantage of needing only one target-validated antibody to develop custom assays. This immunoassay flexibility enables multi-attribute analysis in the same Simple Western instrument and even within the same Simple Western run. With a single platform, Simple Western measures several CQAs for vector characterization. In addition to potency, these include:

- [Purity and identity](#)
- [Empty/full capsid content ratio](#)
- [AAV capsid protein ratio \(VP1:VP2:VP3\)](#)

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17. Ellis *et al* (2013) *Viro J* **10** 74.
18. Eaton *et al* (2013) *PLoS One* **8** e72457.

Appendix

PLA Relative Potency with GraphPad Prism

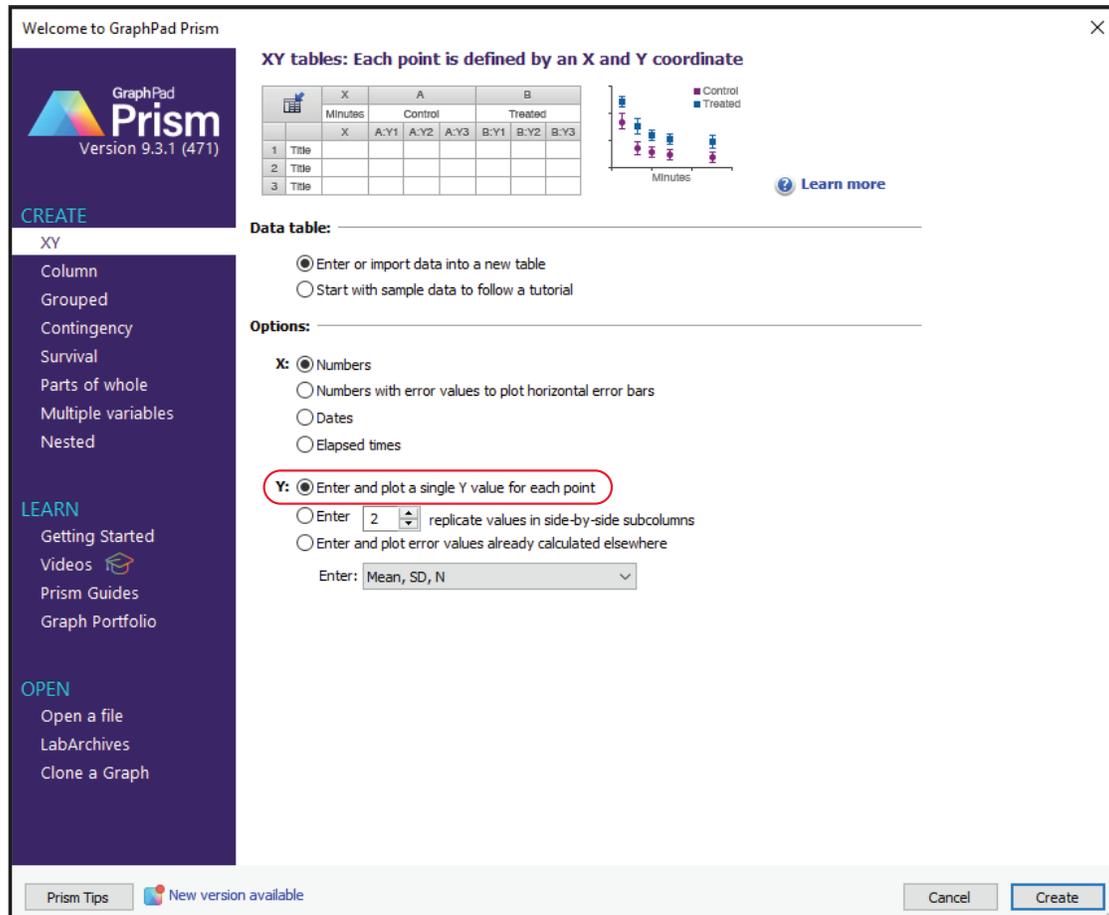
Here, we provide step-by-step instructions on how to determine relative potency by the PLA method using GraphPad Prism analysis software. This PLA method may be applied to calculate relative potency in applications beyond gene therapy, like vaccine development.

At a Glance: Relative Potency Determination

- Transform all data to log
- Apply simple linear regression fit
- Determine linear regression equation for AAV9 Reference and AAV2 Test
- Software determines if the slopes are equal, parallelism
- Solve for X at an overlapping Y value
- Determine relative potency from X value with the following steps:
 - a. Transform $\log(X)$ values to 10^x
 - b. Reference = Reference X-value/Reference X-value
 - c. Test = Reference X-value/Test X-value

Additional details and screenshots are provided below.

1. Create an XY file and select the appropriate Y option, click Create



Welcome to GraphPad Prism

GraphPad Prism
Version 9.3.1 (471)

CREATE

- XY
- Column
- Grouped
- Contingency
- Survival
- Parts of whole
- Multiple variables
- Nested

LEARN

- Getting Started
- Videos
- Prism Guides
- Graph Portfolio

OPEN

- Open a file
- LabArchives
- Clone a Graph

XY tables: Each point is defined by an X and Y coordinate

	X	A			B		
	Minutes	Control			Treated		
	X	A:Y1	A:Y2	A:Y3	B:Y1	B:Y2	B:Y3
1	Title						
2	Title						
3	Title						

Data table:

Enter or import data into a new table
 Start with sample data to follow a tutorial

Options:

X: Numbers
 Numbers with error values to plot horizontal error bars
 Dates
 Elapsed times

Y: Enter and plot a single Y value for each point
 Enter replicate values in side-by-side subcolumns
 Enter and plot error values already calculated elsewhere

Enter:

Prism Tips  New version available

Cancel Create

2. Under **Data Tables**, **Data 1** paste the Reference and Test data

SW Demo Relative Potency Assay.pzfx:Data 1 - GraphPad Prism 9.4.1 (681)

File Edit View Insert Analyze Change Arrange Family Window Help

Prism File Sheet Undo Clipboard Analysis Change Imp

Search...

▼ Data Tables

▣ Data 1

+ New Data Table...

▼ Info

📄 Project info 1

+ New Info...

▼ Results

+ New Analysis...

Table format: XY		X	Group A	Group B	Group C
		Dilution (1/X)	Reference	Test	Title
	X	Y	Y	Y	Y
1	Title	0.00022700	1947271.000	2250161.000	
2	Title	0.00011400	414236.800	545937.700	
3	Title	0.00005680	98082.070	127941.400	
4	Title	0.00002840	21262.260	30716.610	
5	Title	0.00001420	5069.666	8335.324	
6	Title	0.00022700	1847492.000	2587731.000	
7	Title	0.00011400	418913.000	590864.000	

*Ensure that the replicates are stacked in the same column

3. After entering data, click on the **Analyze** button

Select **Transform** in the **Analyze Data** window.

Click **OK**

Analyze

Table format: XY	X	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H	Group I	Group J	Group K
	Dilution (1/X)	Reference	Test	Title	Title	Title	Title	Title	Title	Title	Title	Title
	X	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
1	Title	0.00022700	1947271.000	2250161.000								
2	Title	0.00011400	414236.800	545937.700								
3	Title	0.00005680	98082.070	127941.400								
4	Title	0.00002840	21262.260	30716.610								
5	Title	0.00001420	5069.666	8335.324								
6	Title	0.00022700	1847492.000	2587731.000								
7	Title	0.00011400	418913.000	590864.000								
8	Title	0.00005680	96026.440	134009.600								
9	Title	0.00002840	25228.120	35273.460								
0	Title	0.00001420	6182.289	8895.637								
1	Title											
2	Title											
3	Title											
4	Title											
5	Title											
6	Title											
7	Title											

Analyze Data

Use: Built-in analysis

Which analysis? Search...

Analyze which data sets? Table: Data 1

A:Reference

B:Test

Transform

Transform concentrations (X)

Normalize

Prune rows

Remove baseline and column math

Transpose X and Y

Fraction of total

XY analyses

Nonlinear regression (curve fit)

Simple linear regression

Simple logistic regression

Fit spline/LOWESS

Smooth, differentiate or integrate curve

Area under curve

Deming (Model II) linear regression

Row statistics

Correlation

Interpolate a standard curve

Column analyses

Select All Deselect All

Help Cancel OK

4. In the **Parameters: Transform** window, check the **Transforms X and Y values using X and Y=Log(X or Y)**

The **New Graph – Create a new graph of the results** box should also be selected

Click **OK**

The screenshot shows the GraphPad Prism interface with a data table and the 'Parameters: Transform' dialog box open. The data table has the following content:

	X	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H	Group I	Group J	G
	Dilution (1/X)	Reference	Test	Title	Title	Title	Title	Title	Title	Title	Title	
	X	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	
1	Title	0.00022700	1947271.000	2250161.000								
2	Title	0.00011400	414236.800	545937.700								
3	Title	0.00005680	98082.070	127941.400								
4	Title	0.00002840	21262.260	30716.610								
5	Title	0.00001420	5069.666	8335.324								
6	Title	0.00022700	1847492.000	2587731.000								
7	Title	0.00011400	418913.000	590864.000								
8	Title	0.00005680	96026.440	134009.600								
9	Title	0.00002840	25228.120	35273.460								
10	Title	0.00001420	6182.289	8895.637								
11	Title											
12	Title											
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22	Title											
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27	Title											
28	Title											
29	Title											

The 'Parameters: Transform' dialog box is open, showing the following settings:

- Function List:**
 - Standard functions
 - Pharmacology and biochemistry transforms
 - User-defined X functions
 - User-defined Y functions
- Interchange X and Y (then transform as specified below).
- Transform X values using **X=Log(X)**
- Transform Y values using **Y=Log(Y)**
- Same K for all data sets. K = []
- Different K for each data set
- Data set: Reference K = []
- When it is impossible to transform a SD or SEM:**
 - Erase SD or SEM.
 - Convert to an asymmetric 95% confidence interval.
- Replicates:**
 - Transform individual Y values
 - Transform the average of replicates
- New graph:**
 - Create a new graph of the results

Buttons: Learn, Cancel, OK

6. In the **Parameters: Simple Linear Regression** window, click on the **Compare** option

Also **Calculate** boxes should already be checked

Click **OK**

The screenshot shows the GraphPad Prism interface with a data table and a dialog box. The data table has the following content:

	X	A	B	C	D	E	F	G	H	I	J
	Dilution (1/X)	Reference	Test								
1	-3.644	6.289	6.352								
2	-3.943	5.617	5.737								
3	-4.246	4.992	5.107								
4	-4.547	4.328	4.487								
5	-4.848	3.705	3.921								
6	-3.644	6.267	6.413								
7	-3.943	5.622	5.771								
8	-4.246	4.982	5.127								
9	-4.547	4.402	4.547								
10	-4.848	3.791	3.949								

The 'Parameters: Simple Linear Regression' dialog box is open, showing the following options:

- Interpolate:** Interpolate unknowns from standard curve
- Compare:** Test whether slopes and intercepts are significantly different
- Graphing options:** Show the 95% confidence bands of the best-fit line; Residual plot
- Constrain:** Force the line to go through X= 0, Y= 0
- Replicates:** Consider each replicate Y value as an individual point; Only consider the mean Y value of each point
- Also calculate:** Test departure from linearity with runs test; 95% confidence interval of Y when X = 0; 95% confidence interval of X when Y = 0
- Range:** Start regression line at: Auto; End regression line at: Auto
- Output:** Show this many significant digits (for everything except P values): 4; P value style: GP: 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0
- Make these choices as default for future regressions

Buttons at the bottom: More choices..., Learn, Cancel, OK

7. The Simple linear regression results will appear in the **Results – Simple linear regression** tab.

Under **Tabular results**, you will find the linear regression **Equations** needed to determine the Relative Potency of the Test Sample

SW Demo Relative Potency Assay.pzfx:Simple linear regression of Transform of Data 1 - GraphPad Prism 9.4.1 (681)

File Edit View Insert Analyze Change Arrange Family Window Help

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Search... **Tabular results** x | Are lines different? x | v |

v Data Tables »
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 Data 1
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 + New Graph...

v Layouts »
 + New Layout...

Family
 Data 1
 Transform
 Simple linear regression
 Transform of Data 1

Simple linear regression		A	B
Tabular results		Reference	Test
1	Best-fit values		
2	Slope	2.097	2.036
3	Y-intercept	13.90	13.79
4	X-intercept	-6.629	-6.770
5	1/slope	0.4768	0.4910
6			
7	Std. Error		
8	Slope	0.02397	0.02283
9	Y-intercept	0.1023	0.09741
10			
11	95% Confidence Intervals		
12	Slope	2.042 to 2.152	1.984 to 2.089
13	Y-intercept	13.67 to 14.14	13.56 to 14.01
14	X-intercept	-6.695 to -6.567	-6.838 to -6.705
15			
16	Goodness of Fit		
17	R squared	0.9990	0.9990
18	Sy.x	0.03228	0.03074
19			
20	Is slope significantly non-zero?		
21	F	7652	7956
22	DFn, DFd	1, 8	1, 8
23	P value	<0.0001	<0.0001
24	Deviation from zero?	Significant	Significant
25			
26	Equation	Y = 2.097*X + 13.90	Y = 2.036*X + 13.79
27			
28	Data		
29	Number of X values	10	10
30	Maximum number of Y replicates	1	1
31	Total number of values	10	10
32	Number of missing values	0	0

8. In the same **Results** folder, under the **Are lines different?** tab you will find whether the Reference and Test slopes are equal

GraphPad Prism 9.4.1 (681) - Results window: **Are lines different?**

Are the slopes equal?
 F = 3.364. DFn = 1, DFd = 16
 P=0.0853

If the overall slopes were identical, there is a 8.532% chance of randomly choosing data points with slopes this different. You can conclude that the differences between the slopes are not quite significant.

Since the slopes are not significantly different, it is possible to calculate one slope for all the data. The pooled slope equals 2.067.

Are the elevations or intercepts equal?
 F = 88.80. DFn = 1, DFd = 17
 P<0.0001

If the overall elevations were identical, there is a less than 0.01% chance of randomly choosing data points with elevations this different. You can conclude that the differences between the elevations are extremely significant.

9. Prism compares slopes of two or more regression lines if you check the option: “Test whether the slopes and intercepts are significantly different”. If selected, a summary of this analysis will be presented on the results tab titled “Are lines different?”

Comparing slopes

Prism compares slopes first. It calculates a P value (two-tailed) testing the null hypothesis that the slopes are all identical (the lines are parallel). The P value answers this question:

If the slopes really were identical, what is the chance that randomly selected data points would have slopes as different (or more different) than you observed.

If the P value is less than 0.05

If the P value is low, Prism concludes that the lines are significantly different. In that case, there is no point in comparing the intercepts. The intersection point of the two lines is:

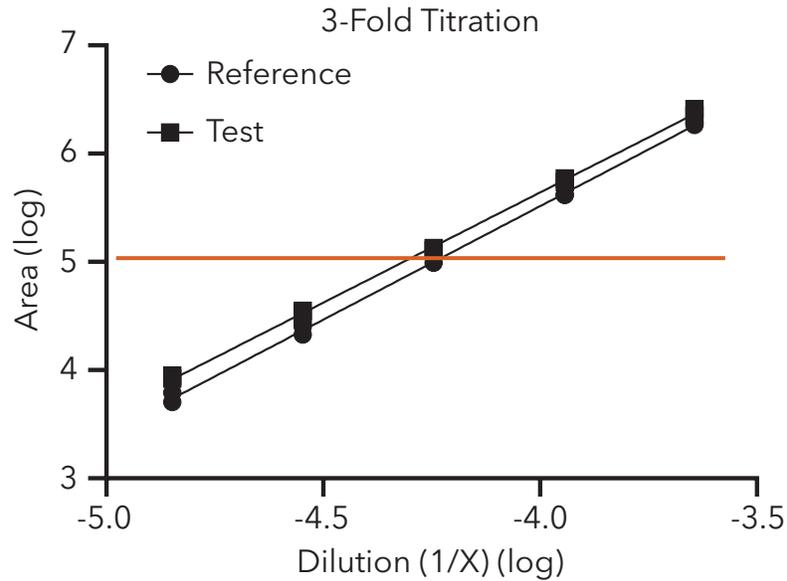
$$X = \frac{\text{Intercept}_1 - \text{Intercept}_2}{\text{Slope}_2 - \text{Slope}_1}$$

$$Y = \text{Intercept}_1 + \text{Slope}_1 \cdot X = \text{Intercept}_2 + \text{Slope}_2 \cdot X$$

If the P value for comparing slopes is greater than 0.05

If the P value is high, Prism concludes that the slopes are not significantly different and calculates a single slope for all lines. Essentially, it shares the Slope parameter between the two data sets.

10. Using the Simple linear regression equations, calculate the Relative Potency by solving each equation for X by using a common Y value(s) between the Reference and Test linear regression curves



11. Determine relative potency from X value with the following steps:

- Transform $\log(X)$ values to 10^x
- Reference = Reference X-value/Reference X-value
- Test = Reference X-value/Test X-value

	reference	test
Slope	2.097	2.036
Y-intercept	13.9	13.79
Rel Pot at log		5
log (X)	-4.24416	-4.31729
power10 (X)	5.7E-05	4.82E-05
	reference	test
Rel Potency	1.00	1.18