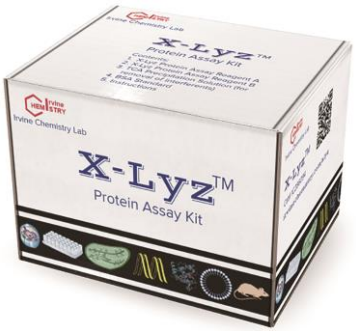
User Instructions

X-Lyz™ Protein Quantitation Assay

Catalog # IC286094-2



Features:

- ***15min quick assay***
- ***No need to calibrate each time***
- ***Wide linear range up to 10,000µg/mL***
- ***Low protein variations***
- ***Compatibility with reducing agents***

For safely handling the reagents, refer to the MSDS sheets online:
www.x-lyz.com

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1 Product Description

The X-Lyz™ Protein Assay Kit is an amine-reactive reagent for the quantitative determination of total proteins or peptides. The water-compatible reagent reacts with the primary amines in proteins or peptides to form a sensitive chromophore; its absorbance at wavelength between 350-405nm is linearly proportional to the protein concentrations over a wide range (10-10,000ug/mL). The method is suitable for assays in microplates and test tubes (cuvettes).

The X-Lyz™ Protein Assay Kit, Catalog # IC286094-2, is sufficient for up to 6400 assays in microplates and 960 assays in test tubes. The contents are specified in the table below.

Table 1: Contents in X-Lyz™ Assay Kit (Cat No. IC286094-2)

Items	Cat No.	Size	Contents	Store	Ship
Reagent A	IC286091	400mL	Containing Reagent A in aqueous solution, pH ~10.5	Room Temp	Room Temp
Reagent B	IC286092	80mL	Containing Reagent B in organic mixture	4ºC	
TCA Precipitation Solution	IC286093	50mL	Containing trichloroacetic acid in aqueous solution, strong acid	Room Temp	
BSA Standard	IC286090	8mL	Containing BSA 20mg/mL in 0.9% saline, 0.05% sodium azide	4ºC	
Instructions	Pub286095	N/A	Instructions for X-Lyz™ Protein Assay Kit	N/A	N/A

All reagents expire one year from the date of purchase.

The assay simply involves mixing the Working Reagent (WR, prepared by combining reagent A and B at a ratio of 5/1) with samples, incubating for 15min and taking measurement at any wavelength between 350-405nm. The WR/Sample ratio of 10/1 and 1/1 may be used for different concentration ranges. A solid protein sample can also be analyzed upon dissolution in the WR.

2 Preparation of Standards and Blank

Blank and diluted BSA standards will be used to generate a standard curve. Use Table 2 & 3 as a guide to prepare the Blank and diluted BSA standards. Starting with the provided BSA standard (20mg/mL, Cat No. IC286090), dilute into the lower concentrations, preferably using the same diluent as the sample(s). The Blank contains only the diluent without BSA. The volumes shown in the tables are sufficient for three replications of each dilution.

Table 2: Preparation of Diluted BSA Standards for WR/Sample Ratio 10/1 Assays

Vials	Diluent (µL)	BSA Sources		Diluted BSA Standards	
		Sources	Volume (µL)	Concentration (µg/mL)	Volume (µL)
1	440	20 mg/mL	440	10000	355
2	225	Vial 1	525	7000	350
3	300	Vial 2	400	4000	355
4	345	Vial 3	345	2000	355
5	335	Vial 4	335	1000	355
6	315	Vial 5	315	500	350
7	280	Vial 6	280	250	350
8	140	Vial 7	210	150	350
Blank	350	none	0	0	350

Table 3: Preparation of Diluted BSA Standards for WR/Sample Ratio 1/1 Assays

Vials	Diluent (µL)	BSA Sources		Diluted BSA Standards	
		Sources	Volume (µL)	Concentration (µg/mL)	Volume (µL)
1	4437	20 mg/mL	493	2000	1600
2	1110	Vial 1	3330	1500	1610
3	1415	Vial 2	2830	1000	1605
4	880	Vial 3	2640	750	1600
5	960	Vial 4	1920	500	1600
6	1280	Vial 5	1280	250	1600
7	960	Vial 6	960	125	1600
8	1280	Vial 7	320	25	1600
Blank	1600	none	0	0	1600

Note: To obtain more accurate assay results, a same type of protein standard as the unknown may be used; for example, a bovine gamma globulin (BGG) standard may be selected for assays of immunoglobulin samples.

3 Preparation of Working Reagent (WR)

1. Use the following formula to determine the total volume of WR required:

   Total Volume of WR Required = (Blank + # of Standards + # of Unknowns) x (Volume of WR per Replicate) x (# of Replicates) x 1.05 (5% extra)

2. Prepare WR by mixing five parts of Reagent A and one part of Reagent B. Use the WR as soon as possible; sitting too long may cause loss of activity.

For example, to conduct an assay of one blank, eight standards and three unknown samples, if 150µL WR is required each in three replicates,

Total Volume of WR Required = (1 + 8 + 3) x 150µL x 3 x 1.05 = 5670µL

The required amount of WR is prepared by mixing 4725µL Reagent A and 945µL Reagent B.

4 Assay Procedures

WR/Sample ratio of 10/1 or 1/1 may be used. The 10/1 ratio gives a wider analytical range, while 1/1 is a more sensitive method. For sample concentration estimation using the amine content in proteins, see Estimation of Concentration by Amine.

Note: For the general estimation of sample concentrations, modern plate readers or spectrophotometers normally have built-in software that automatically plots a linear or nonlinear regression line through the standard points, interpolates the samples, and reports the calculated values. Microsoft Excel may also be used to generate the standard curves, equations and estimate protein samples.

4.1 Microplate Assay Procedure

4.1.1 WR/Sample 10/1 in Microplate

The procedure has a linear range of 150-10,000µg/mL for BSA

1. Pipette 25µL of each Blank, standard and sample into a well.
   Note: Different sample sizes may be used as long as the WR/Sample is 10/1. If the sample size is small, e.g. 10µL, it is recommended to use half-area microplates to maintain the detection sensitivity.
2. Add 250µL WR into each well and shake gently. Cover the plate with parafilm.
3. Incubate for at least 15min at temperature between 25-37°C.
4. Remove parafilm and measure UV absorbance in a plate reader at wavelength between 350-405nm.
5. Subtract the average absorbance of the Blank from that of all other individual standard and unknown sample replicates. The Blank has to be measured every time alongside with the sample(s).
6. Generate a standard curve by plotting the Blank-corrected absorbance of each BSA standard vs. its concentration, and estimate the concentration of each unknown sample.

4.1.2 WR/Sample 1/1 in Microplate

The procedure has a linear range of 25-2,000µg/mL for BSA

1. Pipette 75µL of each Blank, standard and sample into a well.
2. Add 75µL WR into each well and shake gently. Cover the plate with parafilm.
   Note: Different sample sizes may be used, e.g. 125µL, as long as the WR/Sample is 1/1.
3. Incubate for at least 15min at temperature between 25-37ºC.
4. Remove parafilm and measure UV absorbance in a plate reader at wavelength between 350-405nm.
5. Subtract the average absorbance of the Blank from that of all other individual standard and unknown sample replicates. The Blank has to be measured every time alongside with the sample(s).
6. Generate a standard curve by plotting the Blank-corrected absorbance of each BSA standard vs. its concentration, and estimate the concentration of each unknown sample.

4.2 Test Tube Assay Procedure

4.2.1 WR/Sample 10/1 in Test Tube

1. Use Table below to choose the wavelength for a preferred linear analytical range.

Table 4: Analytical Ranges for WR/Sample 10/1

350nm	**370nm**	**390nm**	**405nm**
Analytical Ranges (µg/mL BSA)			
50-2500	50-2500	50-7500	125-7500

2. Pipette 0.1mL of each Blank, standard and sample into the test tube or cuvette.
   Note: Different sample sizes may be used, e.g. 0.25mL, as long as the WR/Sample ratio is 10/1.
3. Add 1mL WR into each test tube and gently mix.
4. Incubate for at least 15min at temperature between 25-37ºC.
5. Transfer to cuvette and measure UV absorbance at the selected wavelength.
   Note: The test sample should be placed in the spectrophotometer chamber right before reading; continuous exposure to UV may affect the absorbance.
6. Subtract the average absorbance of the Blank from that of all other individual standard and unknown sample replicates. The Blank has to be measured every time alongside with the sample(s).

7. Generate a standard curve by plotting the Blank-corrected absorbance of each BSA standard vs. its concentration, and estimate the concentration of each unknown sample.

4.2.2 WR/Sample 1/1 in Test Tube

1. Use Table below to choose the wavelength for a preferred linear analytical range.

Table 5: Analytical Ranges for WR/Sample 1/1

350nm	370nm	390nm	405nm
Analytical Ranges (µg/mL BSA)			
10-500	25-500	25-1000	25-1500

2. Pipette 0.5mL of each Blank, standard and sample into the test tube or cuvette.
   Note: Different sample sizes may be used, e.g. 1mL, as long as the WR/Sample ratio is 1/1.
3. Add 0.5mL WR into each test tube and gently mix.
4. Incubate for at least 15min at temperature between 25-37ºC.
5. Transfer to cuvette and measure UV absorbance at the selected wavelength.
   Note: The test sample should be placed in the spectrophotometer chamber right before reading; continuous exposure to UV may affect the absorbance.
6. Subtract the average absorbance of the Blank from that of all other individual standard and unknown sample replicates. The Blank has to be measured every time alongside with the sample(s).
7. Generate a standard curve by plotting the Blank-corrected absorbance of each BSA standard vs. its concentration, and estimate the concentration of each unknown sample.

5 Compatibility and Interferent Removal

The X-Lyz™ Protein Assay is compatible with reducing agents, common detergents, alcohols, urea, guanidine, metal chelators, and inorganic salts. When high concentrations of these substances are present, however, it is recommended to include them in the standards and Blank.

Primary amine-containing substances, such as amino acids, ammonia, and TRIS buffer interfere with the assay and is recommended to be removed. Non-primary amine buffers may be used to replace TRIS, such as HEPES, BICINE, BIS-TRIS, BIS-TRIS propane, and BIS-TRIS methane.

If necessary, follow the precipitation procedure below to remove the interferents and conduct assay.

1. Thoroughly mix an equal volume of the TCA precipitation solution with a protein sample for one minute in a centrifuge tube or a microplate (a microplate centrifuge is required). The recommended sample volume is 1mL or less.
2. Centrifuge the mixture (6,000 rpm/2,000 x *g* or higher) for 10min, decant and discard the supernatant.
3. Repeat Steps 1 and 2 one more time to obtain a protein precipitate.
4. Dissolve the precipitate in a proper amount of WR, the volume in which the protein concentration should be no more than 700µg/mL.
5. Incubate for at least 15min at temperature between 25-37ºC.
6. Read UV absorbance at wavelength between 350-405nm.
7. The standard curve is generated using the procedure with WR/Sample 10/1 as described in the section Assay Procedures. Estimate a Nominal Concentration by the standard curve.
   Note: Do not precipitate the BSA standards; use them directly to generate the standard curve.
8. Use the formula below to calculate the concentration of the original protein sample.
   Sample Concentration = Nominal Concentration (Step 7) ÷ 11 x WR Volume (Step 4) ÷ Sample Volume (Step 1)

6 Estimation of Concentration by Amine

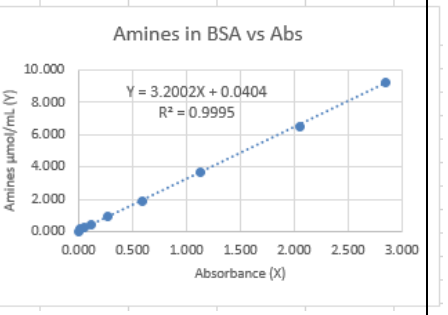
Protein sample concentrations are commonly estimated relative to the mass concentration of the standards. Since the amine-reactive X-Lyz™ assay detects the primary amines present in proteins, the amine content can also be used to calculate the sample concentrations. The approach affords a more accurate estimation with a smaller variation among different proteins as shown in the section Protein Variations.

To estimate sample concentrations by amine, the standard curve of the amine concentration in the BSA standards is plotted. The amine concentrations of the unknown samples are calculated from the standard curve and then converted into the final mass concentrations. In Figure 1, for example, the amine standard curve is generated and the linear equation Y = 3.2002X + 0.0404 is obtained, where Y = amine concentrations and X = absorbance. If an unknown sample of immunoglobulin gives an absorbance of 0.500 (X), its amine concentration (Y) is calculated be 1.641µmol/mL (Y = 3.2002 * 0.500 + 0.0404 = 1.641µmol/mL). The immunoglobulin's molecular weight is ~150,000, and contains 94 primary

amines, the final mass concentration is 2619µg/mL (1.641µmol/mL ÷ 94 * 150,000 = 2619µg/mL).

Figure 1: Standard Curve of Amine Concentration vs. Absorbance of BSA Standards. Axis X is the average of absorbance in five replicates at 350nm; Y is the primary amine concentration in the standards.

	A	B	C
1	Abs (X)	BSA µg/mL	Amine (Y) µmol/mL
2	0.000	0	0.000
3	0.020	150	0.139
4	0.050	250	0.231
5	0.123	500	0.462
6	0.268	1,000	0.924
7	0.589	2,000	1.848
8	1.128	4,000	3.697
9	2.054	7,000	6.470
10	2.845	10,000	9.242
11			
12			



7 Linearity, Ranges and Estimates

The X-Lyz™ assay covers a wide linear range of protein concentrations. Figure 2 shows a linear quantitation range from 150 to 10,000 µg/mL for BSA. The estimation of the protein samples from the standard curve has an excellent agreement with the true values as shown in Table 6.

Figure 2: Standard Curves of BSA from 150-10,000µg/mL at 350nm. Analysis was conducted at 350nm with sample size 25 µL and WR/Sample 10/1 in 96 well microplate.

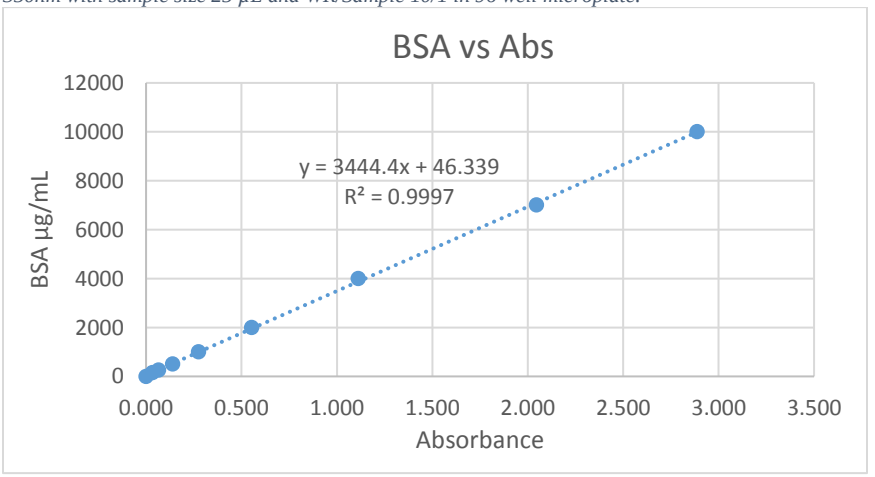


Table 6: Estimates of BSA Samples from the Standard Curve in Figure 2

True Values (µg/mL)	Average Absorbance	Estimates (µg/mL)
200	0.049	215.1
300	0.074	301.2
600	0.160	597.4
900	0.260	941.9
1,900	0.562	1982.1
5,000	1.406	4889.2
9,000	2.600	9001.8

True Values: The true concentrations of BSA samples in µg/mL.
Average Absorbance: Average absorbance in five replicates at 350nm.
Estimates: Estimates of sample concentrations from the standard curve.

8 Protein Variations

The X-Lyz™ assay has shown a low level of variation among different proteins. Relative to BSA mass concentration (µg/mL), the assay results among the tested proteins (Table 7) showed a CV of 21%. When the primary amine concentration is used for calculation, the resultant variation is reduced to CV 8.7% (Table 8). For the detailed procedure using the amines to calculate the results, see the section Estimation of Concentration by Amine.

Table 7: Variation Based on Mass

Proteins	**Ratio vs BSA**
BSA	1.00
BGG	0.76
Ovalbumin	0.69
β-Lactoglobulin	1.09
Trypsin	1.13
Average	0.93
Standard Deviation	0.20
Coefficient of Variation	21%

Table 8: Variation Based on Amine

Proteins	**Ratio vs BSA**
BSA	1.00
BGG	1.00
Ovalbumin	1.20
β-Lactoglobulin	1.16
Trypsin	1.04
Average	1.08
Standard Deviation	0.09
Coefficient of Variation	8.7%

9 Calibration Frequency

Calibration is time-consuming, that requires an assay of a series of standards, and generation of standard curve and equation. The X-Lyz™ assay has demonstrated a highly repeatable performance and does not require to generate standard curve each time. To ensure the completion of color development, it is recommended to incubate 15min at 37ºC for standard curve generation and sample analysis.

However, a new standard curve is required when any of the following events takes place.

- A new type of microplates or cuvettes is used.
- The existing standard curve is longer than six months old.

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