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GFP SimpleStep ELISA® Kit

For the quantitative measurement of GFP in cell and tissue extracts.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Abcam's GFP *in vitro* SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of GFP protein in cell and tissue extracts.

The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB substrate is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at 600 nm.

Green fluorescent protein (GFP) is a 27 kDa protein derived from the jellyfish Aequorea victoria, which emits green light (emission peak at a wavelength of 509 nm) when excited by blue light (excitation peak at a wavelength of 395 nm). GFP has become an invaluable tool in cell biology research, since its intrinsic fluorescence can be visualized in living cells. GFP fluorescence is stable under fixation conditions and suitable for a variety of applications. GFP has been widely used as a reporter for gene expression, enabling researchers to visualize and localize GFP-tagged proteins within living cells without the need for chemical staining. Other applications of GFP include assessment of protein interactions through the yeast two hybrid system and measurement of distance between proteins through fluorescence energy transfer (FRET) protocols. GFP technology has considerably contributed to a greater understanding of cellular physiology. YFP differs from GFP due to a mutation at T203Y; antibodies raised against full-length GFP should also detect YFP and other variants.

2. Protocol Summary

Remove appropriate number of antibody coated well strips. Equilibrate all reagents to room temperature. Prepare the reagents, samples, and standards as instructed.

Add standard or sample to appropriate wells.

Add Antibody Cocktail to all wells. Incubate at room temperature.

Aspirate and wash each well. Add TMB Substrate to each well and incubate. Add Stop Solution at a defined endpoint.

Alternatively, record color development kinetically after TMB substrate addition.

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at +4°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage Condition
10X GFP Capture Antibody	600 µL	4°C
10X GFP Detector Antibody	600 µL	4°C
GFP Lyophilized Recombinant Protein	2 Vials	4°C
4X Antibody Diluent EB	6 mL	4°C
10X Wash Buffer PT	20 mL	4°C
5X Cell Extraction Buffer PTR	10 mL	4°C
50X Cell Extraction Enhancer Solution	1 mL	4°C
TMB Substrate	12 mL	4°C
Stop Solution	12 mL	4°C
Sample Diluent NS	12 mL	4°C
Pre-Coated 96 Well Microplate (12 x 8 well strips)	96 Wells	4°C
Plate Seal	1	4°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- PBS (1.4 mM KH2PO4, 8 mM Na2HPO4, 140 mM NaCl, 2.7 mM KCl, pH 7.4).
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

8. Technical Hints

- This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 11).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.
- When generating positive control samples, it is advisable to change pipette tips after each step.
- The provided 5X Cell Extraction Buffer contains phosphatase inhibitors. Protease inhibitors can be added if required.
- The provided 50X Cell Extraction Enhancer Solution may precipitate when stored at + 4°C. To dissolve, warm briefly at + 37°C and mix gently. The 50X Cell Extraction Enhancer Solution can be stored at room temperature to avoid precipitation.

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. **The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.**
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

9.1 1X Cell Extraction Buffer PTR

Prepare 1X Cell Extraction Buffer PTR by diluting 5X Cell Extraction Buffer PTR and 50X Cell Extraction Enhancer Solution to 1X with deionized water. To make 10 mL 1X Cell Extraction Buffer PTR combine 7.8 mL deionized water, 2 mL 5X Cell Extraction Buffer PTR and 200 µL 50X Cell Extraction Enhancer Solution Mix thoroughly and gently. If required protease inhibitors can be added.

Alternative – Enhancer may be added to Cell Extraction Buffer after extraction of cells or tissue. Refer to note in Troubleshooting section.

9.2 1X Wash Buffer PT

Prepare 1X Wash Buffer PT by diluting 10X Wash Buffer PT with deionized water. To make 60 mL 1X Wash Buffer PT combine 6 mL 10X Wash Buffer PT with 54 mL deionized water. Mix thoroughly and gently.

Δ Note Sufficient volume of 10X Wash Buffer is provided to generate a total volume of 200 mL 1X Wash Buffer PT. Please scale the volume of buffer prepared to match the number of strips being used in the assay.

9.3 1X Antibody Diluent EB

Prepare 1X Antibody Diluent EB by diluting 4X Antibody Diluent EB with 1X Wash Buffer PT. To make 4 mL 1X Antibody Diluent EB combine 1 mL 4X Antibody Diluent EB with 3 mL 1X Wash Buffer PT.

9.4 Antibody Cocktail

Prepare Antibody Cocktail by diluting the capture and detector antibodies in 1X Antibody Diluent EB. To make 3 mL of the Antibody Cocktail combine 300 µL 10X Capture Antibody and 300 µL 10X Detector Antibody with 2.4 mL 1X Antibody Diluent EB. Mix thoroughly and gently.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).

10.1 Reconstitute the GFP protein standard by adding 250 µL water by pipette. Mix thoroughly and gently. Hold at room temperature for 10 minutes and mix gently. This is the 4,000 pg/mL Stock Standard Solution at (see table below).

10.2 Label eight tubes, Standards 1– 8.

10.3 Add 150 µL 1X Cell Extraction Buffer PTR into tube number 1 and 200 µL of 1X Cell Extraction Buffer PTR into numbers 2 8.

10.4 Use the Stock Standard to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:

Standard #	Dilution Sample	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	Stock	150	150	4,000	2,000
2	Standard#1	100	200	2,000	666.7
3	Standard#2	100	200	666.7	222.2
4	Standard#3	100	200	222.2	74.1
5	Standard#4	100	200	74.1	24.7
6	Standard#5	100	200	24.7	8.2
7	Standard#6	100	200	8.2	2.7
8	None	0	200	0	0

11. Sample Preparation

TYPICAL SAMPLE DYNAMIC RANGE	
Sample Type	Range
GFP spiked into 0.5 mg/mL 143B cell lysate	25 – 2,000 pg/mL

11.1 Preparation of extracts from cell pellets

11.1.1 Collect non-adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 5 minutes at 4°C.

11.1.2 Rinse cells twice with PBS.

11.1.3 Solubilize pellet at $2x10^7$ cell/mL in chilled 1X Cell Extraction Buffer PTR.

11.1.4 Incubate on ice for 20 minutes.

11.1.5 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.1.6 Transfer the supernatants into clean tubes and discard the pellets.

11.1.7 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.1.8 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

11.2 Preparation of extracts from adherent cells by direct lysis (alternative protocol)

11.2.1 Remove growth media and rinse adherent cells 2 times in PBS.

11.2.2 Solubilize the cells by addition of chilled 1X Cell Extraction Buffer PTR directly to the plate (use 750 µL - 1.5 mL 1X Cell Extraction Buffer PTR per confluent 15 cm diameter plate).

11.2.3 Scrape the cells into a microfuge tube and incubate the lysate on ice for 15 minutes.

11.2.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.2.5 Transfer the supernatants into clean tubes and discard the pellets.

11.2.6 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.2.7 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

11.3 Preparation of extracts from tissue homogenates

11.3.1 Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).

11.3.2 Homogenize 100 to 200 mg of wet tissue in 500 µL - 1 mL of chilled 1X Cell Extraction Buffer PTR. For lower amounts of tissue adjust volumes accordingly.

11.3.3 Incubate on ice for 20 minutes.

11.3.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.3.5 Transfer the supernatants into clean tubes and discard the pellets.

11.3.6 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.3.7 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

12. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents
- Unused well strips should be returned to the plate packet and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or "edge effects" have not been observed with this assay.

13. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.

13.1 Prepare all reagents, working standards, and samples as directed in the previous sections.

13.2 Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.

13.3 Add 50 µL of all samples and standards to appropriate wells.

13.4 Add 50 µL of the Antibody Cocktail to each well.

13.5 Seal or cover plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.

13.6 Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid.

13.7 Add 100 µL of TMB Substrate to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.

13.8 Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.

Alternative to 13.7 – 13.8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed time in the microplate reader prepared with the following settings:

Mode:	Kinetic
Wavelength:	600 nm
Time:	up to 15 min
Interval:	20 sec - 1 min
Shaking:	Shake between readings

Δ Note *that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 µL Stop Solution to each well and recording the OD at 450 nm.*

13.9 Analyze the data as described below.

14. Calculations

14.1 Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values.

14.2 Create a standard curve by plotting the average blank control subtracted absorbance value for each standard concentration (y-axis) against the target protein concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.

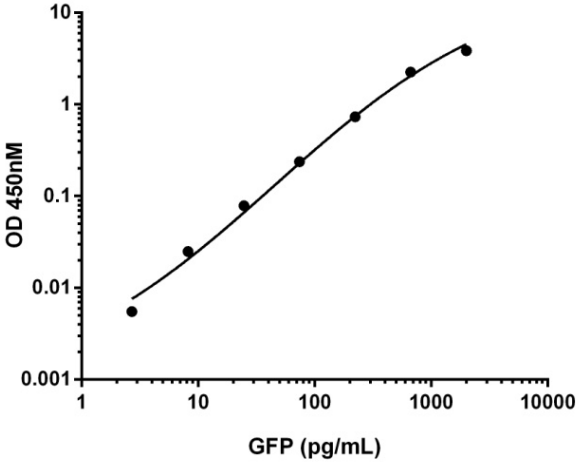
Δ Note Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4 parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.

14.3 Determine the concentration of the target protein in the sample by interpolating the blank control **subtracted** absorbance values against the standard curve**.** Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.

14.4 Samples generating absorbance values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance values less than that of the lowest standard should be retested in a less dilute form.

15. Typical Data

Typical standard curve – data provided **for demonstration purposes only.** A new standard curve must be generated for each assay performed.



Standard Curve Measurements			
Conc. (pg/mL)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.05	0.05	0.05
2.7	0.06	0.06	0.06
8.2	0.08	0.08	0.08
24.7	0.13	0.12	0.12
74.1	0.28	0.27	0.27
222.2	0.73	0.74	0.74
666.7	2.24	2.21	2.23
2,000	3.88	3.86	3.87

Figure 1. Example GFP standard curve. The GFP standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

16. Typical Sample Values

SENSITIVITY –

The calculated minimal detectable (MDD) dose is 1.8 pg/mL. The MDD was determined by calculating the mean of zero standard replicates (n=21) and adding 2 standard deviations then interpolating the corresponding concentrations.

RECOVERY –

(Sample spiking in representative sample matrices)

Sample Type	Average % Recovery	Range %
50% Cell Culture Media	102	101-103
10% FBS	97	96-98

LINEARITY OF DILUTION –

GFP spiked 143B lysate Dilution	GFP spiked 143B lysate (pg/mL)	% Expected Value
undiluted	744.1	100
1:3	248.0	100
1:9	86.6	105
1:27	31.3	114

PRECISION –

Mean coefficient of variations of interpolated values from 3 concentrations of human heart homogenates within the working range of the assay.

	Intra-Assay	Inter-Assay
n=	9	3
CV (%)	3.1	8.2

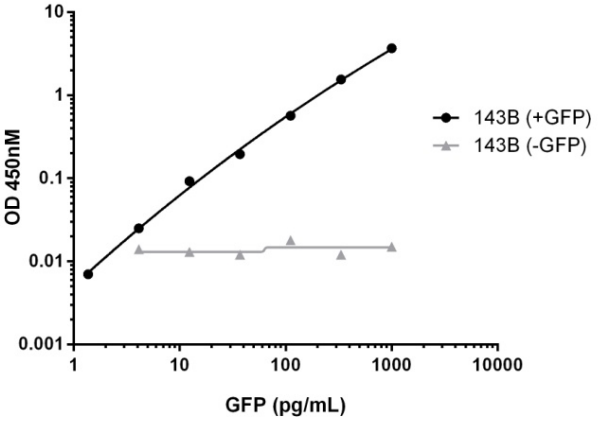


Figure 2. Titration of GFP spiked 143B cell lysate within the working range of the assay. Background subtracted data from triplicate measurements are plotted.

17. Assay Specificity

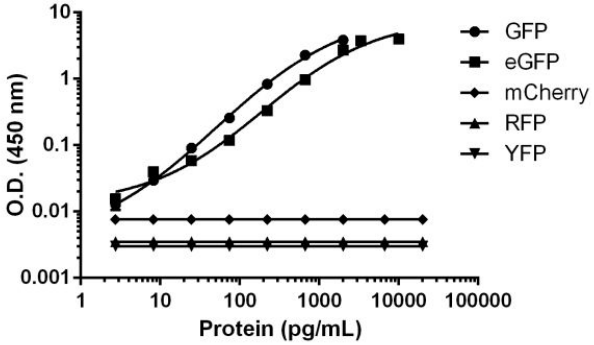


Figure 3. This kit recognizes both GFP and enhanced GFP (eGFP) in cell and tissue extracts. No reactivity with mCherry, RFP or YFP was observed.

Please contact our Technical Support team for more information.

18. Troubleshooting

Problem	Reason	Solution
Difficulty pipetting lysate; viscous lysate.	Genomic DNA solubilized	Prepare 1X Cell Extraction Buffer PTR (without enhancer). Add enhancer to lysate after extraction.
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; increase to 2 or 3 hour standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Incubation times with TMB too brief	Ensure sufficient incubation time until blue color develops prior addition of Stop solution
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your reconstituted standards at -80°C, all other assay components 4°C. Keep TMB substrate solution protected from light.
Precipitate in Diluent	Precipitation and/or coagulation of components within the Diluent.	Precipitate can be removed by gently warming the Diluent to 37°C.

19. Notes

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