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Indirect ELISA protocol (abcam)

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1 Works for me dx.doi.org/10.17504/protocols.io.bn8umhww

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ABSTRACT

The protocol is based on the abcam Indirect ELISA protocol.

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GUIDELINES

General Notes

1.Plate preparation (coating with antigen) takes 12 – 24 hours. Blocking takes 2 – 24 hours. The ELISA assay will take about 5 – 6 additional hours to run, depending on sample prep and number of samples.

2.Run at least 2 replicates for each sample and standard.
3.Allow all reagents to come to room temperature before beginning, at least 15 – 30 minutes.

4.Create plate layout before beginning the assay. Follow the plate layout for placement of standards and samples, and use as a template for the plate reader. If using strip-well plates, remove extra strips that are not needed and store in sealed bag at temperature directed by Ancillary Kit datasheet (2-8^{o}C) or other kit/plate instructions if not using the Ancillary kit.

Example plate layout:

	Strip 1	Strip 2	3	4
A	Standard 1	Standard 1	Sample 1	Sample 1
B	Standard 2	Standard 2	Sample 2	Sample 2
C	Standard 3	Standard 3	Sample 3	Sample 3
D	Standard 4	Standard 4	Sample 4	Sample 4
E	Standard 5	Standard 5	Sample 5	Sample 5
F	Standard 6	Standard 6	Sample 6	Sample 6
G	Standard 7	Standard 7	Sample 7	Sample 7
H	Reagent Blank	Reagent Blank	Sample 8	Sample 8

Optimization of standards, sample dilutions and antibody dilutions:

Because a kit is not being used, the protein standards, sample dilutions and antibody dilutions will need to be optimized by running a test plate.

1. Determine best range for creating standards from pure protein sample, prepare standards accordingly. Use at least 4 standards and up to 7 to create standard curve.
2. Pick 2 to 4 samples to test different dilutions, using the same DFs for each sample:

a.Optimize sample dilution factor: Previous protein assays showed 1:15 and 1:20 DFs to be optimal, so use a range above and below that: 1:20 and 1:50, and/or 1:100 dilutions.

3. Optimize antibody dilution factor for both primary and secondary antibodies. Check datasheets for each antibody to determine dilution range, use at least two dilutions for each antibody.

a.Most datasheets indicate an optimal dilution for the antibody, so start with that DF, then choose a more dilute DF to test as well. (eg Primary Ab DFs at 1:30 and 1:300, secondary Ab DFs at 1:2000 and 1:3000)

4. Organize the plate so that each standard and each sample and sample DF is tested with each antibody DF. The example used for PARP1 indirect ELISA:

i.Sample 1 DF 1:20 test with Primary Ab DF 1:30 + Secondary Ab DF 1:2000
ii.Sample 1 DF 1:50 test with Primary Ab DF 1:30 + Secondary Ab DF 1:2000
iii.Sample 1 DF 1:20 test with Primary Ab DF 1:300+ Secondary Ab DF 1:2000
iv.Sample 1 DF 1:50 test with Primary Ab DF 1:300+ Secondary Ab DF 1:2000
v.Sample 1 DF 1:20 test with Primary Ab DF 1:30 + Secondary Ab DF 1:3000
vi.Sample 1 DF 1:50 test with Primary Ab DF 1:30 + Secondary Ab DF 1:3000
vii.Sample 1 DF 1:20 test with Primary Ab DF 1:300+ Secondary Ab DF 1:3000
viii.Sample 1 DF 1:50 test with Primary Ab DF 1:300 + Secondary Ab DF 1:3000
ix.Apply same set up for other samples and standards (have one standard curve for each antibody dilution set; from the above example, there would be 4 standard curves since there are 4 combinations of Primary Ab and Secondary Ab dilution factors).

5. Run all samples and standards for each DF and antibody set in duplicate.

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MATERIALS TEXT

Materials provided by user:
Primary antibody: Anti-Human PARP1 Antibody (F-2) (Santa Cruz Biotechnologies, catalog # sc-8007) – mouse monoclonal IgG2a antibody

Secondary antibody: Goat anti-mouse IgG-HRP (Santa Cruz Biotechnologies, catalog # sc-2005)

Protein Standard: Poly (ADP-Ribose) Polymerase 1 (PARP1) protein (antibodies-online.com Inc., catalog # ABIN1741730; original supplier: Tulip BioLabs)

Materials provided in the DuoSet Ancillary Reagent Kit 2 (cat #DY008) – or equivalent material provided by user:

ELISA Plate-Coating Buffer:sterile-filtered 1X PBS, contains no preservatives
Reagent Diluent Concentrate 2 (10X concentration):10% BSA solution
Stop Solution:2N sulfuric acid
Color Reagent A:stabilized hydrogen peroxide
Color Reagent B:stabilized tetramethylbenzidine
Wash Buffer Concentrate (25X concentration):solution of buffered surfactant with preservative
Microplates:5 clear high-binding, flat bottom polystyrene microplates (360 uL/well), consisting of 12 removable strips of 8 wells and frame.
ELISA plate sealers

Other Materials Needed (to be provided by user):

PPE: gloves, safety glasses, lab coats
Pipets:P10, P20, P200, P100, multichannel P100, serological pipets (5 mL and 10 mL)
Pipet tips:
15 mL and 50 mL polypropylene centrifuge tubes
Graduated Cylinders
Glass Bottles
Squirt Bottle (or manifold dispenser or autowasher for wash steps)
Microplate reader set to 450 nm

ABSTRACT

The protocol is based on the abcam Indirect ELISA protocol.

BEFORE STARTING

Plate preparation (coating with antigen) takes 12 – 24 hours. Blocking takes 2 – 24 hours. The ELISA assay will take about 5 – 6 additional hours to run, depending on sample prep and number of samples.

Reagent Preparation

1 **PARP1 Standard:** A seven point standard curve using 2-fold serial dilution in Plate Coating Buffer or 1X PBS is recommended. Prepare 500 uL of high standard (1000 ng/mL) per plate assayed from stock solution.

1.1 Final Concentration for each standard for the seven point curve (from highest to lowest): 1000 ng/mL, 500 ng/mL, 250 ng/mL, 125 ng/mL, 62.5 ng/mL, 31.25 ng/mL and 15.625 ng/mL.

1.2 After preparing 1000 ng/mL standard, add 250 uL of Plate Coating Buffer (or PBS) to 6 other microcentrifuge tubes for 2-fold serial dilution for the other standards; label tubes appropriately. The table below shows the 2-fold serial dilution series to create the rest of the standards.

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Standard Concentration	2-fold Serial Dilution
500 ng/mL standard	250 uL of 1000 ng/mL standard + 250 uL Plate Coating Buffer
250 ng/mL standard	250 uL of 500 ng/mL standard + 250 uL Plate Coating Buffer
125 ng/mL standard	250 uL of 250 ng/mL standard + 250 uL Plate Coating Buffer
62.5 ng/mL standard	250 uL of 125 ng/mL standard + 250 uL Plate Coating Buffer
31.25 ng/mL standard	250 uL of 31.5 ng/mL standard + 250 uL Plate Coating Buffer
15.625 ng/mL standard	250 uL of 31.25 ng/mL standard + 250 uL Plate Coating Buffer

1.3 There should be 250 uL of each of the first 6 standards and 500 uL of the 7th standard when done. This is enough to run two sets of standards. Aliquot into 120 uL amounts and store one set of

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standards at -20^{o}C immediately if not using. Place remaining set of standards on ice until use.

1.4 Do not make more than enough standard for two sets unless using them immediately; degradation of store protein standard is highly likely, and it is best to make new standards every one or two runs.

2 **Sample Dilutions:** Using proteins extracted from tissue via Qiagen All-Prep DNA/RNA/protein Mini kit, make dilutions with 1X concentration Plate Coating Buffer (provided in Ancillary DuoSet kit) or PBS at a final volume of 50 uL per set of replicates to be run:

2.1 A 1:50 dilution has been the optimal dilution; to make 1:50 dilution, dilute 2.2 uL protein sample with 107.8 uL 1X Plate Coating Buffer or PBS for a total volume of 110 uL for each set of replicates, which will allow for 50 uL to be pipetted twice.

2.2 For ease and efficiency of pipetting samples onto ELISA plate, prepare sample dilutions in a 96-well PCR plate; make sure samples are in same order as plate layout.

3 **Wash Buffer:** If crystals have formed in the concentrate, warm to room temperature and mix gently iuntil the crystals have gone into solution.

4 **Reagent Diluent:** Calculate amount of working concentration (1X Reagent Diluent) needed for plate blocking and antibody preparation for both antibodies. Dilute 10X concentrate Reagent Diluent to make enough 1X Reagent Diluent for all preparations plus extra to account for pipetting error.

5 **Substrate:** Immediately prior to step 9 of Assay Procedure, prepare Substrate by mixing Color Reagent A and Color Reagent B in 1:1 ratio. Substrate reagents used are from either the DuoSet Ancillary Reagent Kit 2 (cat #DY008) or Substrate Reagent Pack (R&D Systems cat #DY999), and constitute hydrogen peroxide and TMB solution for detection of HRP conjugated reagents. Read the optical density after adding Stop Solution (2N sulfuric acid) at 450 nm.

Assay Procedure

6 Add 50 uL of sample or standards diluted in Plate Coating Buffer, or an appropriate diluent (PBS), per well. Cover with an adhesive strip and incubate overnight at 4^{o}C.

6.1 In case strips may fall out from frame during washes, number each strip respectively according to plate layout.

6.2 The coating incubation time may require some optimization.

7 Remove the coating solution and wash the plate three times by filling the wells with 200 µl of ELISA Wash Buffer, or PBS.

7.1 The solutions or washes are removed by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel.

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7.2 Complete removal of liquid at each step is essential for good performance.

8 Block plates by adding 200 uL of Reagent Diluent to each well. Incubate at room temperature for a minimum of 2 hours.

9 Wash the plate twice with ELISA Wash Buffer, or PBS.

10 Dilute the Primary Antibody to the working concentration in Reagent Diluent. Immediately coat the microplate with 100 uL per well of the diluted Primary Antibody. Seal the plate and incubate a minimum of 2 hours at room temperature.

10.1 Although 2 hours is usually enough to obtain a strong signal, if a weak signal is obtained, stronger staining will often observed when incubated overnight at 4°C.

11 Wash the plate four times with ELISA Wash Buffer, or PBS.

12 Dilute Secondary Antibody to the working concentration in Reagent Diluent, then coat each well of the microplate with 100 uL of the antibody solution. Cover with a new adhesive strip and incubate 1 – 2 hours at room temperature.

13 Wash the plate four times with ELISA Wash Buffer, or PBS.

14 Add 50 uL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light (cover with aluminum foil if needed).

14.1 Immediately prior to step 9 of Assay Procedure, prepare Substrate by mixing Color Reagent A and Color Reagent B in 1:1 ratio. Reagents are from the DuoSet Ancillary Reagent kit, or R&D Systems Substrate Reagent Pack.

15 Add 50 uL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

15.1 Read plate within 30 minutes of adding Stop Solution.

16 Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings to 450 nm. This subtraction will correct for optical imperfections in the plate. Reading made directly at 450 nm without correction may be higher and less accurate.

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16.1 Microplates are read using the SpectraMax spectrophotometer.

16.2 Standard curves and adjusted concentrations are calculated using the SoftMax Pro 6.5 software for the SpectraMax spectrophotometer.

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