

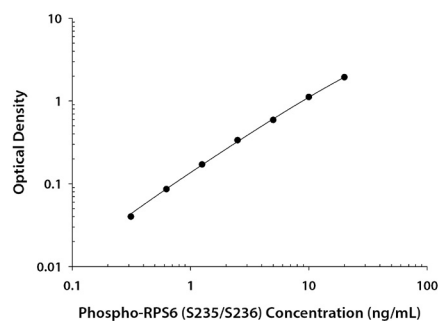
CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample and subtract the average zero standard optical density (O.D.). Results may be normalized to total protein or cell number.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the phospho-RPS6 (S235/S236) concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

TYPICAL DATA

A standard curve should be generated for each set of samples assayed. The graph below represents typical data generated when using this Human/Mouse/Rat Phospho-RPS6 (S235/S236) DuoSet IC ELISA. The standard curve was calculated using a computer generated 4-PL curve-fit. This standard curve is for demonstration purposes only.



CALIBRATION

The Human/Mouse/Rat Phospho-RPS6 (S235/S236) DuoSet IC ELISA is calibrated against a highly purified *E. coli*-expressed recombinant human phospho-RPS6 (S235/S236) produced at R&D Systems®. Samples containing natural phospho-RPS6 (S235/S236) showed linear dilution parallel to the standard curve obtained using the Human/Mouse/Rat Phospho-RPS6 (S235/S236) Standard. These results indicate that O.D. values from this DuoSet IC ELISA can be used to determine the relative concentration of phospho-RPS6 (S235/S236) in natural samples.

QUANTIFICATION

Amounts of phosphorylated human RPS6, as quantified by the Human/Mouse/Rat Phospho-RPS6 (S235/S236) DuoSet IC ELISA, are consistent with the relative amounts of phosphorylated RPS6 determined by qualitative Western Blot analysis.

The Human/Mouse/Rat Phospho-RPS6 (S235/S236) DuoSet IC ELISA also quantifies phosphorylated RPS6 levels in mouse and rat cell lysates.

SPECIFICITY

The Human/Mouse/Rat Phospho-RPS6 (S235/S236) DuoSet IC ELISA specifically recognizes RPS6 dually phosphorylated at S235 and S236. Specificity was demonstrated using both peptide competition and cross-reactivity analysis.

Cross-reactivity experiments were performed with the Human/Mouse/Rat Phospho-RPS6 (S235/S236) DuoSet IC ELISA to further determine specificity. Unphosphorylated recombinant human RPS6 was tested at 200 ng/mL and did not cross-react in the assay.

TECHNICAL HINTS & LIMITATIONS

- This DuoSet IC ELISA should not be used beyond the expiration date on the kit label.
- Individual results may vary due to differences in technique, plasticware, and water sources.
- It is important that the diluents selected for reconstitution and for dilution of the standard and samples reflect the environment of the samples being measured. The diluents suggested in this protocol should be suitable for most cell lysates.
- The type of enzyme and substrate and the concentrations of capture/detection antibodies used can be varied to create an immunoassay with a different sensitivity and dynamic range. A basic understanding of immunoassay development is required for the successful use of these reagents in immunoassays.
- A thorough and consistent wash technique is essential for proper assay performance. Wash Buffer should be dispensed forcefully and removed completely from the wells by aspiration or decanting. Remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- Use a fresh reagent reservoir and pipette tips for each step.
- It is recommended that all standards and samples be assayed in duplicate.
- Avoid microbial contamination of reagents and buffers. This may interfere with the sensitivity of the assay. Buffers containing protein should be made under aseptic conditions and stored at 2-8 °C or be prepared fresh daily.

PRECAUTIONS

The Stop Solution recommended for use with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B recommended for use with this kit may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

DuoSet™ IC ELISA INTRACELLULAR

Human/Mouse/Rat Phospho-RPS6 (S235/S236)

Catalog Number: DYC3918-2 (2 plates)
DYC3918-5 (5 plates)
DYC3918E (15 plates)

INTENDED USE

For the development of sandwich ELISAs to measure human, mouse, and rat Ribosomal Protein S6 (RPS6) phosphorylated at S235 and S236 in cell lysates.

PRINCIPLE OF THE ASSAY

This DuoSet IC ELISA contains the basic components required for the development of sandwich ELISAs to measure human/mouse/rat Ribosomal Protein S6 (RPS6) phosphorylated at S235 and S236 in cell lysates. An immobilized capture antibody specific for human/mouse/rat RPS6 binds both phosphorylated and unphosphorylated protein. After washing away unbound material, a biotinylated detection antibody specific for human/mouse/rat RPS6 dually phosphorylated at S235 and S236 is used to detect only the phosphorylated protein, utilizing a standard Streptavidin-HRP format.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

DESCRIPTION	PART #	CATALOG # DYC3918-2	CATALOG # DYC3918-5	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human/Mouse/Rat Phospho-RPS6 (S235/S236) Capture Antibody	842936	1 vial	2 vials	Store for up to 1 month at 2-8 °C or aliquot and store at ≤ -20 °C for up to 3 months in a manual defrost freezer.*
Human/Mouse/Rat Phospho-RPS6 (S235/S236) Detection Antibody	842937	1 vial	2 vials	
Human/Mouse/Rat Phospho-RPS6 (S235/S236) Standard	842938	3 vials	5 vials	Use within one hour of reconstitution. Use a fresh standard for each assay.
Streptavidin-HRP A	890803	1 vial	1 vial	Store for up to 3 months at 2-8 °C.* DO NOT FREEZE.

* Provided this is within the expiration date of the kit.

DYC3918-2 contains sufficient materials to run ELISAs on at least two 96 well plates.[†]
DYC3918-5 contains sufficient materials to run ELISAs on at least five 96 well plates.[†]

This kit is also available in an Economy Pack (R&D Systems®, Catalog # DYC3918E).
Economy Packs contain sufficient materials to run ELISAs on 15 microplates.[†]
Specific vial counts of each component may vary. Refer to the literature accompanying
your order for specific vial counts.

- [†] Provided the following conditions are met:
- The reagents are prepared as described in this package insert.
 - The assay is run as described in the General ELISA Protocol.
 - The recommended microplates, buffers, diluents, substrates, and solutions are used.

OTHER MATERIALS REQUIRED

- Aprotinin (Tocris™ # 4139)
- Leupeptin (Tocris # 1167)
- Pepstatin (Tocris # 1190)
- NP-40 Alternative (EMD/Calbiochem # 492016)
- Phenylmethylsulfonyl Fluoride (PMSF) (Sigma # P7626)
- Sodium Azide (Na₃N₃) (Sigma # S2002)
- Sodium Deoxycholate (Sigma # D6750)
- Sodium Dodecyl Sulfate (SDS) (Sigma # L4509)
- Sodium Fluoride (NaF) (Sigma # 201154)
- Sodium Orthovanadate (Na₃VO₄) (Sigma # S6508), activated
- Sodium Pyrophosphate (Na₄P₂O₇) (Sigma # P8010)
- Pipettes and pipette tips
- Deionized or distilled water
- 96 well microplates (R&D Systems, Catalog # DY990)
- Plate sealers (R&D Systems, Catalog # DY992)
- Squirt bottle, manifold dispenser, or automated microplate washer

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SOLUTIONS REQUIRED

PBS - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄,
pH 7.2-7.4, 0.2 µm filtered (R&D Systems, Catalog # DY006).

Wash Buffer - 0.05% Tween® 20 in PBS, pH 7.2-7.4 (R&D Systems,
Catalog # WA126).

Block Buffer - 1% BSA, * 0.05% NaN₃, in PBS, pH 7.2-7.4.

IC Diluent #1 - 1% BSA* in PBS, pH 7.2-7.4, 0.2 µm filtered
(R&D Systems, Catalog # DY995).

IC Diluent #15 - 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40
Alternative, 0.5% Sodium Deoxycholate, 0.1% SDS.

Lysis Buffer #10 - 50 mM Tris, 1% NP-40 Alternative, 0.25% Sodium
Deoxycholate, 5 mM EDTA, 150 mM NaCl, 5 mM NaF, 10 µg/mL
Leupeptin, 10 µg/mL Pepstatin, 100 µM PMSF, 3 µg/mL Aprotinin,
2 mM Sodium Pyrophosphate, 1 mM activated Sodium Orthovanadate
in PBS, pH 7.2-7.4.

Substrate Solution - 1:1 mixture of Color Reagent A (H₂O₂) and Color
Reagent B (Tetramethylbenzidine) (R&D Systems, Catalog # DY999).

Stop Solution - 2 N H₂SO₄ (R&D Systems, Catalog # DY994).

*The use of R&D Systems Reagent Diluent Concentrate 2
(Catalog # DY995) or Millipore Bovine Serum Albumin,
Fraction V, Protease free (Catalog # 82-045) is recommended. All buffers
containing BSA must be stored at 2-8 °C.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Human/Mouse/Rat Phospho-RPS6 (S235/S236) Capture Antibody
(Part 842936) - Each vial contains 720 µg/mL of mouse anti-human
RPS6 antibody when reconstituted with 200 µL of PBS.

**Human/Mouse/Rat Phospho-RPS6 (S235/S236) Detection
Antibody** (Part 842937) - Each vial contains 9.0 µg/mL of biotinylated
rabbit anti-human phospho-RPS6 (S235/S236) antibody when
reconstituted with 1.0 mL of IC Diluent #1. Immediately before use,
dilute the detection antibody to a working concentration of
250 ng/mL in IC Diluent #1. Prepare only as much detection antibody
as required to run each assay.

Human/Mouse/Rat Phospho-RPS6 (S235/S236) Standard
(Part 842938) **Reconstitute with a recommended volume of 500 µL
of IC Diluent #15 to produce a stock solution. Refer to the vial
label for the concentration of recombinant human phospho-RPS6
(S235/S236).** A seven point curve using 2-fold serial dilutions and a
high standard of 20 ng/mL is recommended.

Streptavidin-HRP A (Part 890803) - 1.0 mL of Streptavidin conjugated
to horseradish-peroxidase. Immediately before use, dilute the
Streptavidin-HRP A to the working concentration specified on the vial
label using IC Diluent #1.

PREPARATION OF SAMPLES

Cell Lysates - Rinse cells two times with PBS, making sure to remove
any remaining PBS after the second rinse. Solubilize cells at
1 x 10⁷ cells/mL in Lysis Buffer #10 and allow samples to sit on ice for
15 minutes. Assay immediately or store at ≤ -70° C. Before use,
centrifuge samples at 2000 x g for 5 minutes and transfer the
supernate to a clean test tube. Sample protein concentration may be
quantified using a total protein assay. If needed, further dilutions
should be made in IC Diluent #15.

GENERAL ELISA PROTOCOL

Plate Preparation

1. Dilute the capture antibody to a working concentration of
4.0 µg/mL in PBS, without carrier protein. Immediately coat a
96 well microplate with 100 µL per well of the diluted capture
antibody. Seal the plate and incubate overnight at room
temperature.
2. Aspirate each well and wash with Wash Buffer, repeating the
process two times for a total of 3 washes. Wash by filling each
well with Wash Buffer (400 µL) using a squirt bottle, manifold
dispenser, or autowasher. Complete removal of liquid at
each step is essential for good performance. After the last
wash, remove any remaining Wash Buffer by aspirating or by
inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 µL of Block Buffer to each well.
Incubate at room temperature for 1-2 hours.
4. Repeat the aspiration/wash as in step 2. The plates are now
ready for sample addition.

Assay Procedure

1. Add 100 µL of standard and sample in IC Diluent #15 per well.
Use IC Diluent #15 as the zero standard. Cover with a plate
sealer and incubate 2 hours at room temperature.
Note: A seven point standard curve using 2-fold serial dilutions
and a high standard of 20 ng/mL is recommended.
2. Repeat the aspiration/wash as in step 2 of the Plate Preparation.
3. Add 100 µL of the diluted detection antibody to each well.
Cover with a new plate sealer and incubate 2 hours at room
temperature.
4. Repeat the aspiration/wash as in step 2 of the Plate Preparation.
5. Add 100 µL of the diluted Streptavidin-HRP A to each well.
Incubate for 20 minutes at room temperature. Avoid placing
the plate in direct light.
6. Repeat the aspiration/wash as in step 2 of the Plate Preparation.
7. Add 100 µL of Substrate Solution to each well. Incubate for
20 minutes at room temperature. Avoid placing the plate in
direct light.
8. Add 50 µL of Stop Solution to each well. Gently tap the plate to
ensure thorough mixing.
9. 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 at 450 nm. This subtraction will correct for optical
imperfections in the plate. Readings made directly at 450 nm
without correction may be higher and less accurate.