INTRODUCTION

Targeted gene knockdown using small interfering RNA (siRNA) or antisense oligonucleotide has been valuable technology for studying gene function. Gene knockdown leads to reduction in mRNA and subsequently protein expression. It can be often verified at mRNA level by Northern blot or quantitative RT-PCR. However, decrease in the amount of a specific mRNA does not typically correlate well with protein levels present in the cell. Gene knockdown can be also measured at the protein level with Western blot. Western blot analysis is the most comprehensive way of showing that expression of the target gene has been down regulated. However this method, while sensitive, often lacks the ability to discriminate between samples in which the differences in protein levels are minimal. It is also limited in its application to high-throughput analysis. The kit has the following features:

- Quick and efficient. Completion of entire assay needs only 4 hours.
- Innovative colorimetric assay with no need for radioactivity, electrophoresis, and chromatography.
- The internal control is conveniently included to correct for the variations for the cell number or protein concentrations.
- Strip microplate format makes the assay flexible: manual or high throughput.
- Simple, reliable, and consistent assay conditions.

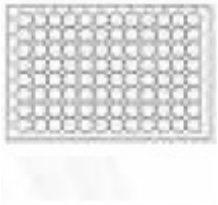
PRINCIPLE AND PROCEDURE

E2F4 Gene Knockdown Quantification Kit For Tumor Sup-pressors/Oncogenes is specifically designed for quantifying gene knockdown induced by siRNA or antisense oligonucleotide at the protein level in the cultured cells or tissues. In the assay, the cell lysates containing the targeted protein are stably spotted on the specifically treated microwells with unique protein capture buffer. The spotted protein can be then recognized with the target-specific antibody and colorimetrically measured through detection antibody-chromogen reaction system.

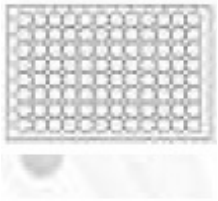
Cell Lysis

Protein extraction

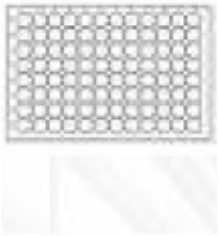
Protein spotted onto assay wells



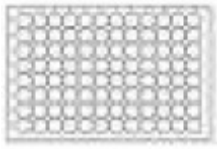
Add capture antibody after wash

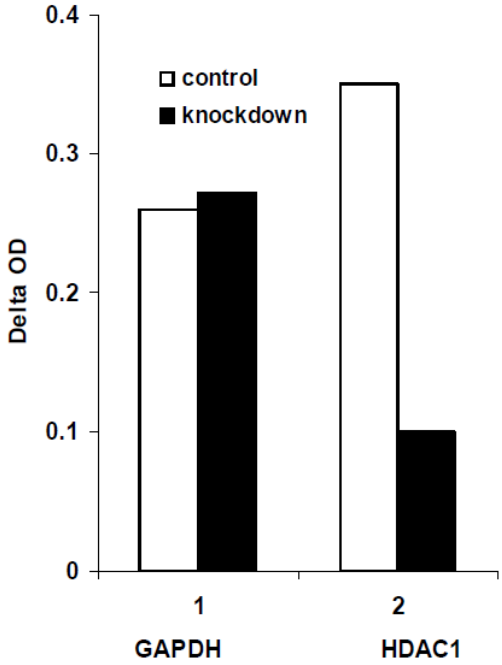


Add detection antibody after wash



Add developing solution for developing color and measure absorbance





Quantification of HDAC1 knockdown. MCF-7 cells were treated or untreated with HDAC1 siRNA. Protein extracts were prepared and used for detection of HDAC1 protein level.

PRODUCT USE INFORMATION

E2F4 Gene Knockdown Quantification Kit For Tumor Sup-pressors/Oncogenes is suitable for quantifying gene knockdown caused by siRNA or antisense oligonucleotides using mammalian tissue and cell extracts.

E2F4 Gene Knockdown Quantification Kit For Tumor Sup-pressors/Oncogenes series offers a flexible choice of different kits used for measuring knockdown of 41 common genes related to tumor Sup-pressors/ oncogenes.

Abnova guarantees the performance of all products in the manner described in our product instructions.

Abnova reserves the right to change or modify any product to enhance its performance and design.

E2F4 Gene Knockdown Quantification Kit For Tumor Sup-pressors/Oncogenes is for research use only and is not intended for diagnostic or therapeutic application.

MATERIAL AMD METHOD

A. List of component

Components	96 assays
Q1 (Extraction Bbuffer)	12 ml
Q2 (10X Wash Buffer)	28 ml
Q3 (Protein Capture Buffer)	1 ml
Q4 (Blocking Buffer)	20 ml
Q5 (Antibody Buffer)	12 ml
Q6 (Developing Solution)	10 ml
Q7 (Stop Solution)	6 ml
GAPDH Control Antibody*	20 µl
Capture Antibody*	25 µl
Detection Antibody*	20 µl
8-Well Assay Strips (with Frame)	12
User Guide	1

* For maximum recovery of the products, centrifuge the original vial prior to opening the cap.

SHIPPING AND STORAGE

The kit is shipped in two parts: one part at ambient room temperature, and the second part on frozen ice packs at 4℃.

Upon receipt: (1) Store the Detection Antibody at –20℃; (2) Store Q2, Q4, Q6, GAPDH Control Antibody, Capture Antibody, and 88-Well Assay Strips at 4℃ away from light; (3) Store all other components at room temperature. The kit is stable for up to 6 months from the shipment date, when stored properly.

Note: Check if wash buffer, Q2 contains salt precipitates before using. If so, warm (at room temperature or 37℃) and shake the buffer until the salts are re-dissolved.

MATERIALS REQUIRED BUT NOT SUPPLIED

Centrifuge
Orbital shaker
Microplate reader
Pipettes and pipette tips
15 conical tubes
1.5 ml microcentrifuge tubes
PBS
Distilled water

PROTOCOL

Protein Extraction

For Adherent Cells:

1. Grow cells (treated or untreated) to 70-80% confluency in 12 well or 6 well plate, trypsinize, and collect cells into 15 ml tube.
2. Centrifuge the cells at 1000 rpm for 5 min and discard the supernatant. Add 1 ml of PBS into the cell pellet, suspend and transfer cells into a 1.5 ml vial. Pellet cells again by centrifuging at 1000 rpm for 5 min.
3. Remove supernatant as much as possible and add **Q1** (40 µl/well for 12 well plate and 100 µl/well for 6 well plate) to re-suspend cell pellet, vortex and incubate on ice for 10 min.
4. Pellet cell debris by centrifuging at 12,000 rpm for 10 min at 4C. Transfer the supernatant to a new vial. At this stage the supernatant can be immediately used or store at –80℃.

Note: For 96 well plate cultures, **Q1** can be directly added into the wells in 5 µl/ well and incubate at room temperature for 5 min to lyse cells. The lysed cell solution is transferred to a 0.5 ml vial and centrifuge at 12,000 rpm for 10 min. Supernatant is transferred to a new 0.5 ml vial for storage or to the strip well for assay (see below).

For Suspension Cells:

1. Collect cells (treated or untreated) into a 15 ml conical tube. Count cells in a hemacytometer.
2. Centrifuge the cells at 1,000 rpm for 5 min and discard the supernatant. Add 1 ml of PBS into the cell pellet, suspend and transfer cells into a 1.5 ml vial. Pellet cells again by centrifuging at 1000 rpm for 5 min.
3. Remove supernatant as much as possible and add **Q1** (50 µl/ 1 x 10^6 cells) to resuspend cell pellet, vortex and incubate on ice for 10 min.
4. Pellet cell debris by centrifuging at 12,000 rpm for 10 min at 4℃. Transfer the supernatant to a new vial. At this stage the supernatant can be immediately used or store at –80℃.

Target Protein Level Detection

1. Determine the number of strip wells required. Leave these strips in the plate frame (remaining unused strips can be placed back in the bag. Seal the bag tightly and store at 4℃). Dilute **Q2** with distilled water (pH 7.2-7.5) at 1:10 ratio.
2. Dilute the protein extract with **Q3** at 1:1 ratio (ex: add 5 µl of **Q3** to 5 µl of protein extracts). Add 10 µl of the diluted protein extract into central area of each strip well. Spread out the solution over the strip well surface by pipetting the solution up and down several times. Incubate the strip wells at 37℃ (with no humidity) for 90 min to evaporate the solution and dry the wells). For blank, add 10 µl of **Q3** instead of protein extract.

   Note: The non-evaporated solution may be gathered along the edges at the bottom of the well. Make sure the well is completely dry by slightly tilting the well and aspirating against the edge with a P-10 or P-20

pipette. If there is still the residue solution, extend incubation time for an additional 15-30 min to dry the well.

3. Add 150 µl of **Q4** to the wells and incubate at 37℃ for 30-45 min.
4. Aspirate and wash the wells with 150 µl of diluted **Q2** three times.
5. Dilute GAPDH control antibody (at 1:100 ratio) to 1 µg/ml with **Q5**. Also dilute the antibodies specific for the target proteins to the appropriate concentration (0.5-1 µg/ml) with **Q5**. Add 50 µl of the diluted GAPDH control antibody and your antibodies to the wells separately and incubate at room temperature for 60 min on an orbital shaker (50-100 rpm).
6. Aspirate and wash the wells with 150 µl of diluted **Q2** four times.
7. Dilute the detection antibody (at 1:1000 ratio) with **Q5**. Add 50 µl of the diluted detection antibody to the well in which GAPDH control antibody was added. Also, dilute the secondary antibodies affinity to the capture antibodies with **Q5** to an appropriate concentration (0.1-0.2 µg/ml). And add 50 µl of secondary antibodies into the wells the capture anti-bodies were added. Incubate at room temperature for 30 min.
8. Aspirate and wash the wells with 150 µl of the diluted **Q2** five times.
9. Add 100 µl of **Q6** to the wells and incubate at room temperature for 2-10 min away from light. Monitor color development in the sample and control wells (blue).
10. Add 50 µl of **Q7** to the wells and read absorbance on microplate reader at 450 nm.
11. Calculate % target protein level:

$$\text{Protein}\,\% = \frac{\text{ODT (treated sample blank)/ODC (untreated control blank)}}{\text{ODT (untreated control blank)/ODC (treated sample blank)}} \text{ x } 100\%$$

Here OD_T is OD value for the target protein. OD_C is OD value for the GAPDH control.

TROUBLESHOOTING

No Signal for the Sample

The protein sample is not properly extracted.	Ensure the protein extraction protocol is suitable for your protein sample preparation.
The protein amount is added into well insufficiently.	Ensure extract contains enough amount of proteins.
Reagents are added incorrectly.	Check if reagents are added in order and if some steps of the procedure are omitted by mistake.
The well is not completely dried.	Ensure the well is incubated with no humidity and dry before adding block buffer.
The well is incorrectly washed before protein spotting.	Ensure the well is not washed before adding protein extracts.
Incubation time and temperature is incorrect.	Ensure the incubation time and temperature described in the protocol are followed correctly.
Protein extracts are incorrectly stored.	Ensure the nuclear extracts are stored at –80℃.

High Background Present for the Blank

The well is not washed enough.	Check if wash at each step is performed according to the protocol.
Insufficient antibody dilution.	Increase antibody dilution.
Overdevelopment.	Decrease development time in step 9 of “target protein level detection.”