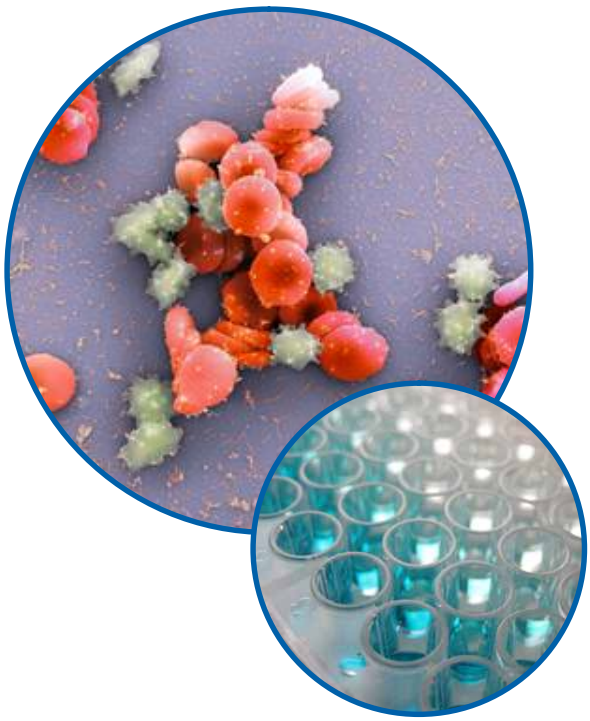

AdipoGen™

MANUAL

NQO1 (human) (IntraCellular) ELISA Kit

For research use only. Not for diagnostic use.

Version 2 (18-March-2011)

Cat. No. AG-45A-0036EK-KI01

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1. Intended Use

The NQO1 (human) (IntraCellular) ELISA Kit is to be used for the *in vitro* quantitative determination of human NQO1 in cell lysates or cell-based assays (screening). This ELISA Kit is for research use only.

2. Introduction

NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1) is a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector (1). NQO1 plays a dual role in protection from carcinogenesis. Its anti-oxidative properties protect the cell from carcinogenic oxidative damage, whereas its ability to stabilize the tumor suppressor p53 protein is important for eliminating damaged cells that are prone to develop cancer. The p53 level is regulated by the rate of its degradation, which is known to be mediated by the Mdm2–ubiquitin–proteasome degradation pathway (2-3). Today accumulated evidence show that there exists also an ubiqutin-independent p53 proteasomal degradation pathway (4). Recent studies link p53 to metabolism, since p53 was shown to influence mitochondrial activity and to inhibit glycolysis. Drastic changes in the cell metabolism are postulated to be a cause for cancer, what is known as the Warburg effect. At high glycolysis levels, when NAD(P)H is high, NQO1 stabilizes and protects p53 from ubiquitin-independent degradation, a process that is NADH dependent, and also elevates NAD+/NADH levels. The NAD+/NADH ratio is an important value in sensing the metabolism dynamics in a cell. Thus NQO1-p53 cross talk is important to regulate p53 protein levels and the metabolic state of the cell. Due to the ability of NQO1 to protect p53 from degradation, it can be considered as a tumor suppressor (5). An allele of NQO1 gene is associated with increased risk of developing different types of tumors (6). NAD+ and NADH play a crucial role in cellular energy metabolism, and a dysregulated NAD+/NADH ratio is implicated in metabolic syndrome (7-8). A recent study has demonstrated that pharmacological activation of NADH oxidation by NQO1 provoked mitochondrial fatty acid oxidation and ameliorated obesity and related phenotypes in mice (9). In humans, NQO1 is expressed at high levels in adipocytes and its expression levels are positively correlated with adiposity, glucose tolerance, and liver dysfunction (10). Thus NQO1 likely has an important role in metababolism and may provide the basis for a new therapy for the treatment of metabolic syndrome.

3. General References

(1) NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector: A.T. Dinkova-Kostova, et al.; Arch. Biochem. Biophys. 501, 116 (2010)

(2) Mdm2 promotes the rapid degradation of p53: Y. Haupt, et al.; Nature 387, 296 (1997)

(3) Regulation of p53 stability by Mdm2: M.H. Kubbutat, et al.; Nature 387, 299 (1997)

(4) Mdm-2 and ubiquitin-independent p53 proteasomal degradation regulated by NQO1: G. Asher, et al.; Proc. Natl. Acad. Sci. 99, 13125 (2002)

(5) Genome wide identification of recessive cancer genes by combinatorial mutation analysis: S. Volinia, et al.; PLoS One 3, e3380 (2008)

(6) NAD(P)H:quinone oxidoreductase 1 (NQO1,DT-diaphorase), functions and pharmacogenetics: D. Ross, et al.; Methods Enzymol. 382, 115 (2004)

(7) The new life of a centenarian: signalling functions of NAD(P): F. Berger, et al.; Trends Biochem. Sci. 29, 111 (2004)

(8) The power to reduce: pyridine nucleotides--small molecules with a multitude of functions: N. Pollak, et al.; Biochem. J. 402, 205 (2007)

(9) Pharmacological stimulation of NADH oxidation ameliorates obesity and related phenotypes in mice: J.H. Hwang, et al.; Diabetes 58, 965 (2009)

(10) The expression of NAD(P)H:quinone oxidoreductase 1 is high in human adipose tissue, reduced by weight loss, and correlates with adiposity, insulin sensitivity, and markers of liver dysfunction: J. Palming, et al.; J. Clin. Endocrinol. Metab. 92, 2346 (2007)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human NQO1 in cells. A monoclonal antibody specific for NQO1 has been precoated onto the 96-well microtiter plate. Standards and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, NQO1 is recognized by the addition of a purified polyclonal antibody specific for NQO1 (Detection Antibody). After removal of excess polyclonal antibody, HRP conjugated anti-rabbit IgG (Detector) is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The intensity of the color reaction is measured at 450 nm after acidification and is directly proportional to the concentration of NQO1 in the samples.

5. Handling & Storage

- Reagent must be stored at 2-8°C when not in use
- Plate and reagents should be at room temperature before use.
- Do not expose reagents to temperatures greater than 25°C.

6. Kit Components

1 plate coated with human NQO1 Antibody	(12 x 8-well strips)
1 bottle Wash Buffer 10X	(50 ml)
1 bottle Diluent 5X	(50 ml)
1 bottle Lysis Buffer 10X	(12 ml)
1 bottle Detection Antibody	(12 ml)
1 vial Detector 100X (HRP Conjugated anti- rabbit IgG)	(150 µl)
1 vial human NQO1 Standard (lyophilized)	(40 ng)
1 vial human NQO1 QC sample (lyophilized)	
1 bottle TMB Substrate Solution	(12 ml)
1 bottle Stop Solution	(12 ml)
3 plate sealers (plastic film)	

7. Materials Required but *Not* Supplied

- Microtiterplate reader at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Calibrated precision single and multi-channel pipettes. Disposable pipette tips
- Deionized water
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working buffers
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard
- Phenyl methylsulfonyl fluoride (PMSF)

8. General ELISA Protocol

8.1. Preparation and Storage of Reagents

NOTE: *Prepare just the appropriate amount of the buffers necessary for the assay.*

- **Wash Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 50 ml Wash Buffer 10X + 450 ml water) to obtain Wash Buffer 1X.
- **Diluent 5X** has to be diluted with deionized water 1:5 before use (e.g. 50 ml Diluent 5X + 200 ml water) to obtain Diluent 1X.
- **Lysis Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 12 ml Lysis Buffer 10X + 108 ml water) to obtain Lysis Buffer 1X. Add 1 mM PMSF immediately before use.
- **Detector 100X (HRP Conjugated anti-rabbit IgG)** has to be diluted to the working concentration by adding 120 µl in 12 ml of Diluent 1X (1:100).

  NOTE: *The diluted Detector is used within one hour of preparation.*

- **Human NQO1 Standard (STD)** has to be reconstituted with 1 ml of deionized water.

  - This reconstitution produces a stock solution of 40 ng/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

  NOTE: *The reconstituted standard is aliquoted and stored at -20°C*

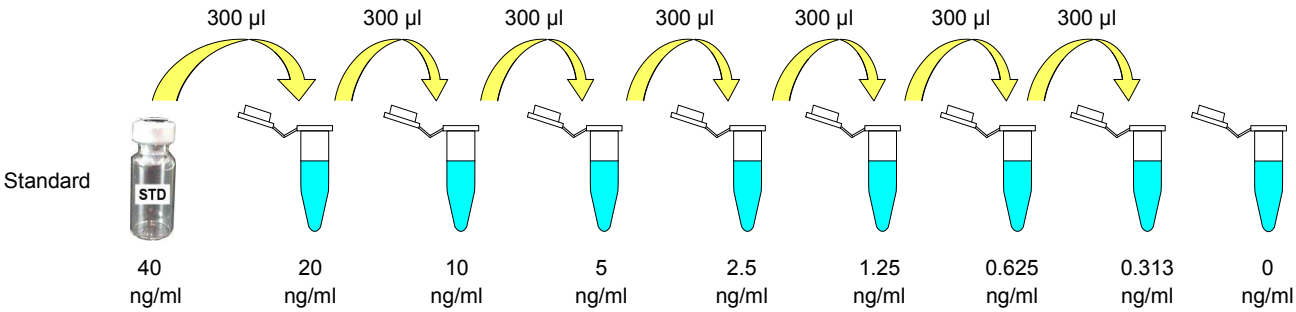
  - Dilute the standard protein concentrate (STD) (**40 ng/ml**) in Diluent 1X. A seven-point standard curve using 2-fold serial dilutions in Diluent 1X is recommended.
  - Suggested standard points are:
    20 , 10 , 5 , 2.5 , 1.25 , 0.625 , 0.313 and 0 ng/ml.

- **Human NQO1 QC sample** has to be reconstituted with 1 ml of deionized water.

  - Refer to the Certificate of Analysis for current QC sample concentration. Mix the QC sample to ensure complete reconstitution and allow the QC sample to sit for a minimum of 15 minutes. The reconstituted QC sample is ready to use, do not dilute it.

Dilute further for the standard curve:

To obtain	Add	Into
20 ng/ml	300 µl of NQO1 (40 ng/ml)	300 µl of Diluent 1X
10 ng/ml	300 µl of NQO1 (20 ng/ml)	300 µl of Diluent 1X
5 ng/ml	300 µl of NQO1 (10 ng/ml)	300 µl of Diluent 1X
2.5 ng/ml	300 µl of NQO1 (5 ng/ml)	300 µl of Diluent 1X
1.25 ng/ml	300 µl of NQO1 (2.5 ng/ml)	300 µl of Diluent 1X
0.625 ng/ml	300 µl of NQO1 (1.25 ng/ml)	300 µl of Diluent 1X
0.313 ng/ml	300 µl of NQO1 (0.625 ng/ml)	300 µl of Diluent 1X
0 ng/ml	300 µl of Diluent 1X	Empty tube



8.2. Sample Collection, Storage and Dilution

Cell Lysates : Grow cell until 90% confluency. Scrap cells off the plate and transfer to an appropriate tube. Keep on ice and microcentrifuge at 1,200 rpm for 5 minutes at 4°C. Remove supernatant, rinse cells once with ice-cold PBS. Remove PBS and add 200 µl ice-cold 1x lysis buffer supplemented with 1 mM phenyl methylsulfonyl fluoride (PMSF) to ten million cells of interest and incubate on ice for 30 minutes. Microcentrifuge at 12,000 rpm for 5 minutes at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Use freshly prepared cell lysate samples.

Cell Lysates have to be diluted in Diluent 1X. Samples containing visible precipitates must be clarified before use.

NOTE: *As a starting point, 1/10 to 1/1,000 dilutions of cell lysates are recommended! If samples fall the outside range of assay, a lower or higher dilution may be required!*

8.3. Assay Procedure (Checklist)

☐	1. Determine the number of 8-well strips needed for the assay and insert them in the frame for current use. The extra strips should be resealed in the foil pouch bag and stored at 4°C. ***NOTE:*** *Remaining 8-well strips coated with NQO1 antibody when opened can be stored at 4°C for up to 1 month.*
☐	2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted lysates samples in duplicate to the wells **(see 8.1. Preparation and Storage of Reagents and 8.2. Preparation of Samples).**
☐	3. Cover the plate with plate sealer and incubate for **1 hour at 37°C.**
☐	4. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of three washes. After the last wash, complete removal of liquid is essential for good performance.
☐	5. Add 100 µl to each well of the Detection Antibody.
☐	6. Cover the plate with plate sealer and incubate for **1 hour at 37°C**.
☐	7. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of three washes. After the last wash, complete removal of liquid is essential for good performance.
☐	8. Add 100 µl to each well of the diluted Detector **(see 8.1. Preparation and Storage of Reagents)**.
☐	9. Cover the plate with plate sealer and incubate for **1 hour at 37°C**.
☐	10. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	11. Add 100 µl to each well of TMB Substrate Solution.
☐	12. Allow the color reaction to develop **at room temperature (RT°C) in the dark for 20 minutes**.
☐	13. Stop the reaction by adding 100 µl of Stop Solution. Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution is added.
	! CAUTION: CORROSIVE SOLUTION!
☐	14. Measure the OD at 450 nm in an ELISA reader within 30 minutes.

9. Calculation of Results

- Average the duplicate readings for each standard, QC and sample and subtract the average blank value (obtained with the 0 ng/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding NQO1 concentration (ng/ml) on the vertical (Y) axis (see **10.** TYPICAL DATA).
- Calculate the NQO1 concentrations of samples by interpolation of the regression curve formula as shown above in a form of a quadratic equation
- If the test samples were diluted, multiply the interpolated values by the dilution factor to calculate the concentration of human NQO1 in the samples.

10. Typical Data

The following data are obtained using the different concentrations of standard as described in this protocol:

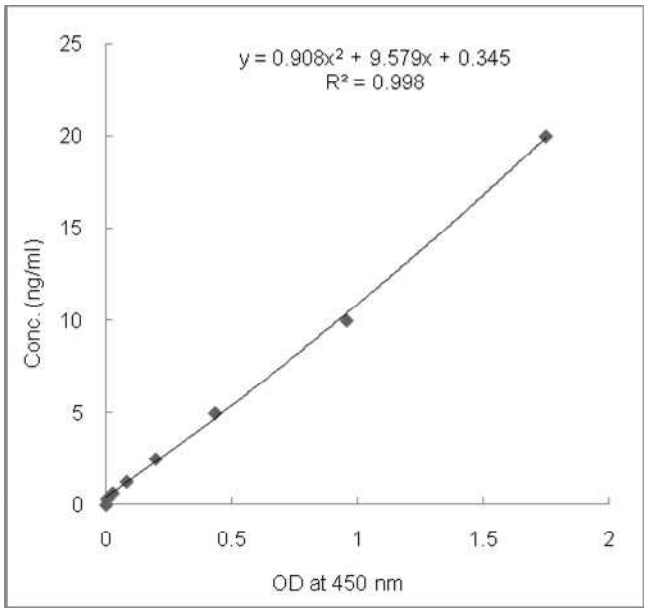


Figure: Standard curve

Standard hNQO1 (ng/ml)	Optical Density (mean)
20	1.752
10	0.958
5	0.434
2.5	0.198
1.25	0.082
0.625	0.026
0.313	0.004
0	0

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of NQO1 that can be detected by this assay is 100 pg/ml. ***NOTE:*** *The Limit of detection was measured by adding two standard deviations to the mean value of 50 zero standard.*

B. Assay range: 0.313 ng/ml – 20 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human NQO1. It does not cross-react with human adiponectin, human RBP4, human Nampt, human vaspin, human progranulin, human resistin, human clusterin, human ANGPTL3, human CTRP5, human IL-33, human leptin, human GPX3, human NMNAT2, human sirtuin 1, human FTO, mouse Nampt, rat Nampt.

D. Intra-assay precision:

Four samples of known concentrations of human NQO1 were assayed in replicates 8 times to test precision within an assay.

Samples	Means (µg/ml)	SD	CV (%)	n
A549 cells	9.156	0.442	4.828	8
HT-29 cells	2.465	0.158	6.424	8
HepG2 cells	1.570	0.115	7.034	8
HeLa cells	4.242	0.108	2.555	8

E. Inter-assay precision:

Four samples of known concentrations of human NQO1 were assayed in 5 separate assays to test precision between assays.

Samples	Means (µg/ml)	SD	CV (%)	n
A549 cells	8.795	0.376	4.276	5
HT-29 cells	3.735	0.269	7.201	5
HepG2 cells	1.628	0.101	6.213	5
HeLa cells	1.147	0.112	9.738	5

F. Recovery:

When samples (cell lysates) are spiked with known concentrations of human NQO1, the recovery averages 96% (range from 90% to 105%).

Samples	Average recovery (%)	Range (%)
A549 cells	98.23	95-105
HT-29 cells	93.05	90-100
HepG2 cells	93.38	90-100
HeLa cells	99.47	95-105

G. Linearity:

Different human cell lysates samples containing NQO1 were diluted several fold (1/200 to 1/3,200) and the measured recoveries ranged from 95% to 110%.

Samples	Sample Dilution	Expected (µg/ml)	Observed (µg/ml)	% of Expected
A549 cells	1 : 800	8.089	8.089	100
	1 : 1,600	4.045	4.335	107.18
	1 : 3,200	2.022	2.180	107.78
HT-29 cells	1 : 200	2.533	2.533	100
	1 : 400	1.266	1.206	95.20
	1 : 800	0.633	0.651	102.76
HepG2 cells	1 : 200	1.269	1.269	100
	1 : 400	0.635	0.640	100.77
	1 : 800	0.317	0.344	108.52
HeLa cells	1 : 200	0.878	0.878	100
	1 : 400	0.439	0.428	97.43
	1 : 800	0.220	0.227	103.46

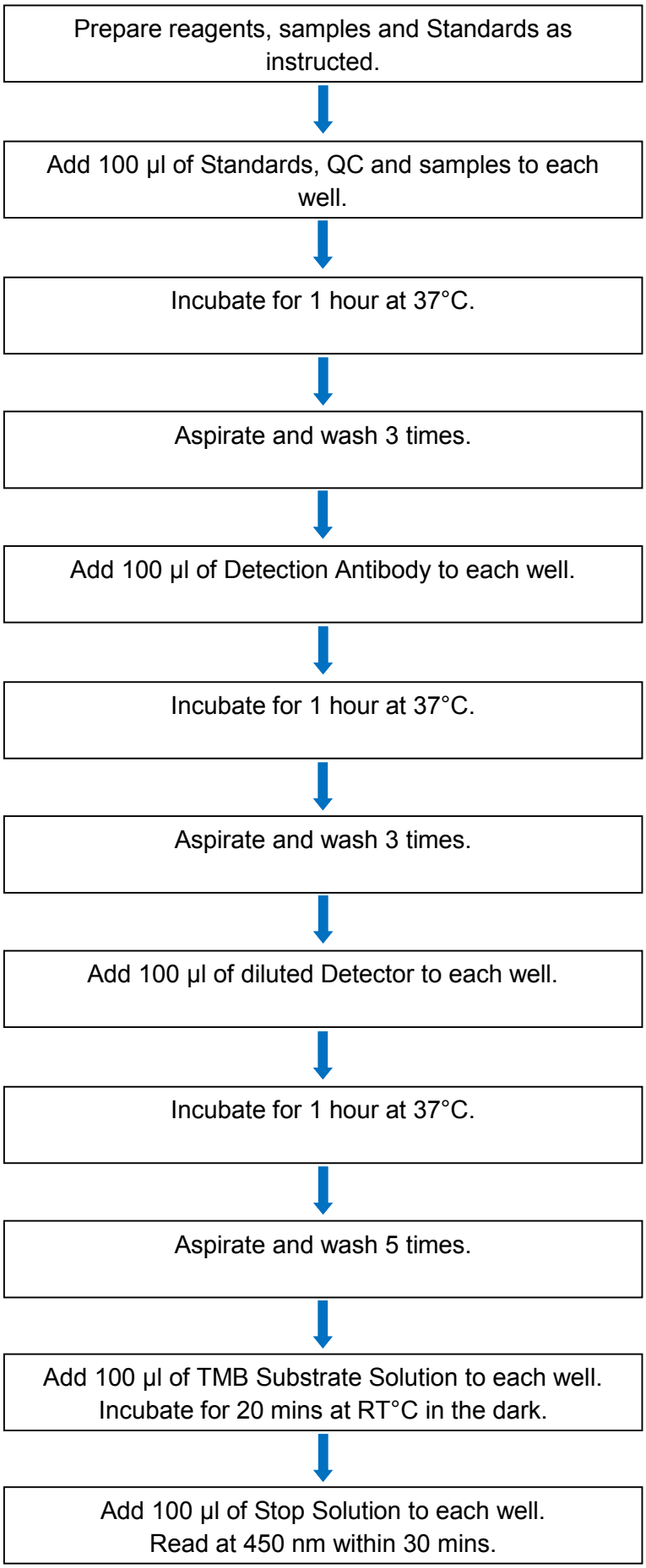
12. Technical Hints and Limitations

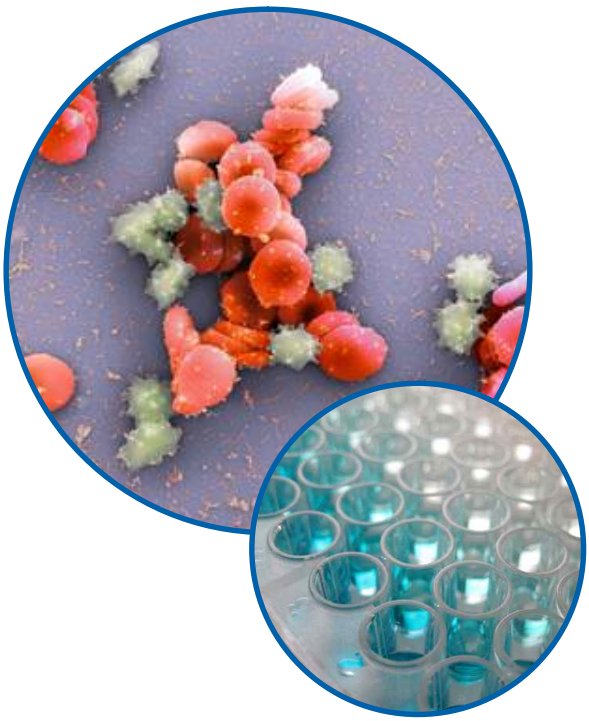
- It is recommended that all standards, QC sample and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100 µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
- Crystals could appear in the 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37°C before dilution of the buffer solutions.
- Once reagents have been added to the 8-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Substrate Solution protected from light.
- The Stop Solution consists of phosphoric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.

13. Troubleshooting

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be followed as indicated in the manual.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of detector too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.

14. Assay Flow Chart





Product Specific References:

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