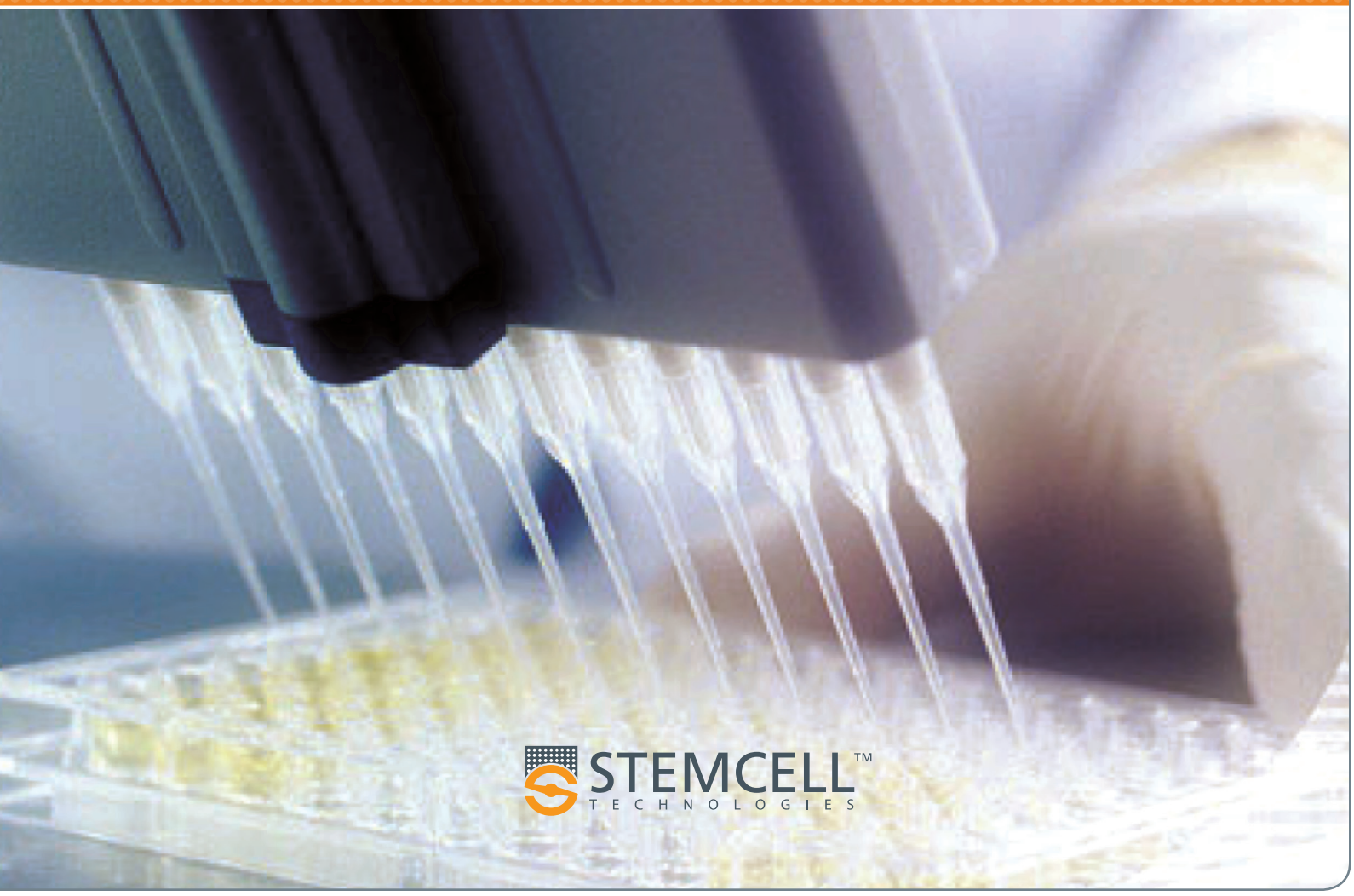
# Human Erythropoietin

## ELISA Kit



STEMCELL™ TECHNOLOGIES

# Table of **Contents**



For the quantitative measurement of natural and recombinant human erythropoietin (EPO) in biological fluids

Flow Chart

# EPO ELISA

Add 25 µL of Buffer A to each well

↓

Add 50 µL of test sample or EPO Standard

↓

Add 50 µL of Biotinylated Anti-EPO Antibody

↓

Seal wells with adhesive cover

↓

Incubate at room temperature for 1 hour on shaker (recommended)
or 2 hours on benchtop

↓

Wash five times with 1X Wash Buffer

↓

Add 100 µL of Streptavidin-HRP Conjugate

↓

Incubate at room temperature for 15 minutes on shaker (recommended)
or 30 - 60 minutes on benchtop

↓

Wash five times with 1X Wash Buffer

↓

Add 100 µL of TMB Substrate Solution

↓

Incubate 15 minutes at room temperature

↓

Add 100 µL of Stop Solution (0.5 N sulphuric acid)

↓

Measure light absorption at 450 nm

**Please read the detailed Assay Procedure on page 7 before performing the ELISA.**

# Human Erythropoietin (EPO)

ELISA Kit

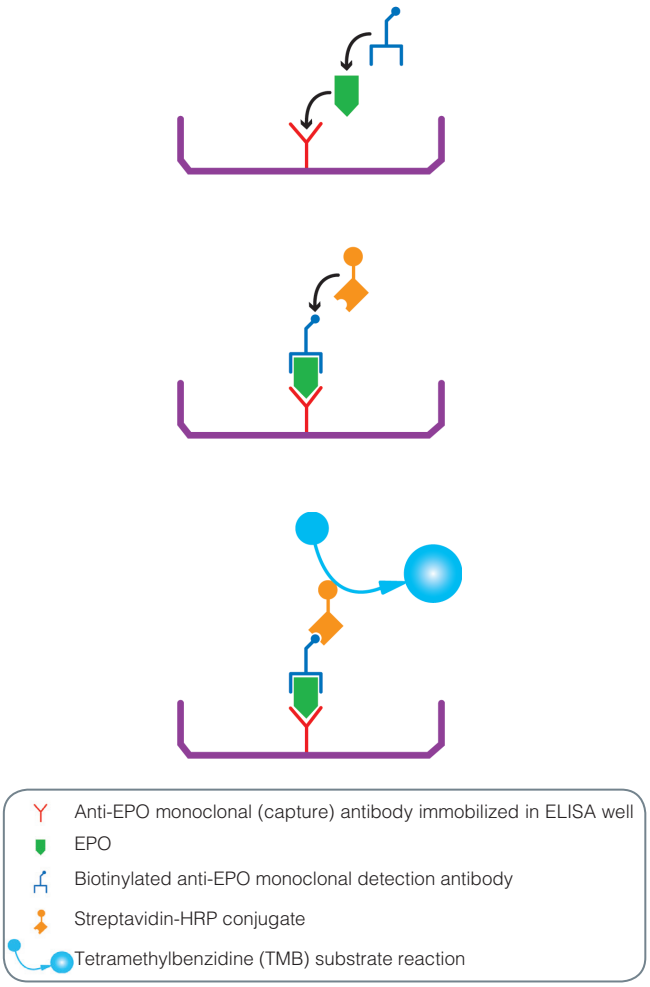
## Intended Use

The STEMCELL Technologies EPO ELISA is designed for the quantitative measurement of natural and recombinant human erythropoietin in biological fluids, such as serum, plasma and tissue culture supernatants. **The ELISA is for Research Purposes Only and is not intended for diagnostic purposes.** Please note that the ELISA is intended for measurement of human EPO only. It has not been validated for detection of EPO from other species.

## Assay Principle

The EPO ELISA utilizes two monospecific monoclonal antibodies raised against human urinary EPO. These antibodies bind two non-overlapping epitopes on the EPO polypeptide and show high-affinity binding to both natural and recombinant EPO. The ELISA is based on the double antibody or sandwich method, and consists of three steps:

1. Test samples or EPO Standards and Biotinylated Anti-EPO Antibody are incubated simultaneously in a 96-well microtiter plate that has been precoated with an anti-EPO monoclonal antibody. During incubation, EPO present in the test sample or standards binds to the immobilized antibody on the plate and the Biotinylated Anti-EPO Antibody binds to the immobilized EPO. Unbound substances are washed away.
2. Wells are incubated with streptavidin conjugated to horseradish peroxidase. During incubation the Streptavidin-Peroxidase Conjugate (streptavidin-HRP), binds to the immobilized biotinylated anti-EPO antibody. Unbound reagents are washed away.
3. Chromogenic substrate is added to the wells and is oxidized by the immobilized peroxidase yielding a blue-colored reaction product. The reaction is stopped with acid, which converts the blue product into yellow. The color intensity is proportional to the amount of EPO present in each well and is determined spectrophotometrically at a wavelength of 450 nm. The EPO concentration of the test sample is determined by comparing the absorbance to a standard curve of the absorbance values of each EPO-standard plotted against the known EPO concentration of each standard.




**Please read the detailed Assay Procedure on page 7 before performing the ELISA.**

## List of Components Provided with Kit

**Microplate.** 96-well plate (12 strips of 8 wells) pre-coated with anti-EPO monoclonal antibody.

**Buffer A.** Phosphate buffered saline containing protein, detergent and preservative (0.09% (vol/vol) Proclin): 3 mL per kit. Buffer A is added to each well at the start of the procedure.

**Buffer B.** Phosphate buffered saline containing protein, detergent and preservative (0.09% (vol/vol) Proclin): 50 mL per kit. Buffer B is provided as a diluent for test samples.

**EPO Standards:**
**(100 mU/mL; 50 mU/mL; 25 mU/mL; 12.5 mU/mL; 6.25 mU/mL; 3.12 mU/mL; 1.56 mU/mL; 0 mU/mL)**
1 mL of each standard per kit. The standards consist of human recombinant EPO serially diluted in Buffer B. The standards have been calibrated against the International Standard for Recombinant DNA-Derived EPO (National Institute for Biological Standards and Control (NIBSC) Code: 87/684 or 11/170).

**Biotinylated Anti-EPO Antibody.** Biotinylated monoclonal antibody against human EPO diluted in Buffer B: 7 mL per kit.

**Streptavidin-Peroxidase Conjugate.** Streptavidin conjugated to horseradish peroxidase (HRP) and diluted in a HRP-stabilizing buffer: 12.5 mL per kit.

**TMB Substrate Solution.** Ready-to-use buffered solution of tetramethylbenzidine (TMB) and hydrogen peroxide: 12.5 mL per kit.

**Stop Solution.** 0.5 N sulphuric acid: 12.5 mL per kit. Attention: Sulphuric acid is corrosive. Handle with care!

**10X Wash Buffer.** Concentrate of phosphate buffered saline containing detergent; sufficient to make 1 L of wash buffer: 100 mL per kit.

**Adhesive Covers.** 4 per kit.

## Materials Required but not Included with Kit

- Pipettes, micropipette, multi-channel pipette, pipette tips
- Reagent trays, lab glassware
- Absorbent tissues
- Distilled or deionized water to prepare 1X Wash Buffer from 10X Wash Buffer
- 1 L graduated measuring cylinder and 1 L bottle to prepare 1X Wash Buffer from 10X Wash Buffer
- Microplate spectrophotometer set at wavelength of 450 nm and (optional) a second correction wavelength at or above 600 nm
- Microplate shaker (recommended)
- Multi-channel plate washer (optional)
- Vortex mixer (optional)

## Precautions for Use

1. Distilled or deionized water must be used for reagent preparation. Water deionized with polyester resins may inactivate the horseradish peroxidase enzyme. Store the water in non-metallic containers.
2. Only use clean or disposable glass or plasticware for preparation of the wash buffer.
3. Cross-contamination between reagents may invalidate the assay results. Permanently labeled, dedicated, multi-channel micropipette reservoirs for the appropriate reagents are recommended.
4. Use TMB in adequately ventilated areas. Avoid contact with skin. Eye irritation may result from direct eye contact.
5. To achieve accurate results, each well must be thoroughly washed during the washing steps.

## Test Sample Preparation

***Serum.*** Collect blood in a clot tube or use a serum separator. Allow samples to coagulate (30 minutes at room temperature) and centrifuge for 15 minutes at 700 - 800 x *g*. Collect the serum and dispense into aliquots of 150 µL or more. A minimum of 100 µL is needed per duplicate test of undiluted sample. Store at -20°C if the samples are not used the same day. Avoid repeated freezing and thawing of the same sample.

***Plasma.*** Collect blood in tubes containing anticoagulants. Centrifuge samples at 700 - 800 *x g*. Collect the plasma and dispense into aliquots of 150 µL or more. A minimum of 100 µL is required per duplicate test of undiluted sample. Store at -20°C if the samples are not used the same day. Avoid repeated freezing and thawing of the same sample.

***Tissue culture supernatant.*** Centrifuge samples at 700 - 800 x *g* to pellet cells. Collect the supernatant and store at 2 - 8°C until use. If the samples are not used the same day, it is recommended to dispense the supernatants into aliquots of 150 µL and store at -20°C. Avoid repeated freezing and thawing of the same sample.

***Note: Human blood, serum and plasma samples pose potential biohazards and should be handled and disposed of using appropriate safety precautions.***

***Before each ELISA:*** Thaw frozen test samples and mix the contents thoroughly by vortexing. Remove any particulate matter by centrifugation or filtration prior to use. Samples can be tested either undiluted (50 µL per well) or after dilution in Buffer B (supplied). EPO levels in samples containing over 100 mU/mL can only be measured accurately after dilution. Although EPO concentrations can be obtained from a single sample (tested in duplicate) it is advisable to test several different dilutions of the same sample, especially for samples with a high EPO concentration. This increases the likelihood that one of the measurements is within the range of the assay (1.6 - 100 mU/mL). Avoid using lipemic, hemolysed or contaminated samples as these may yield unreliable results.

## Assay Procedure

(Time: Approximately 2 - 3 hours)

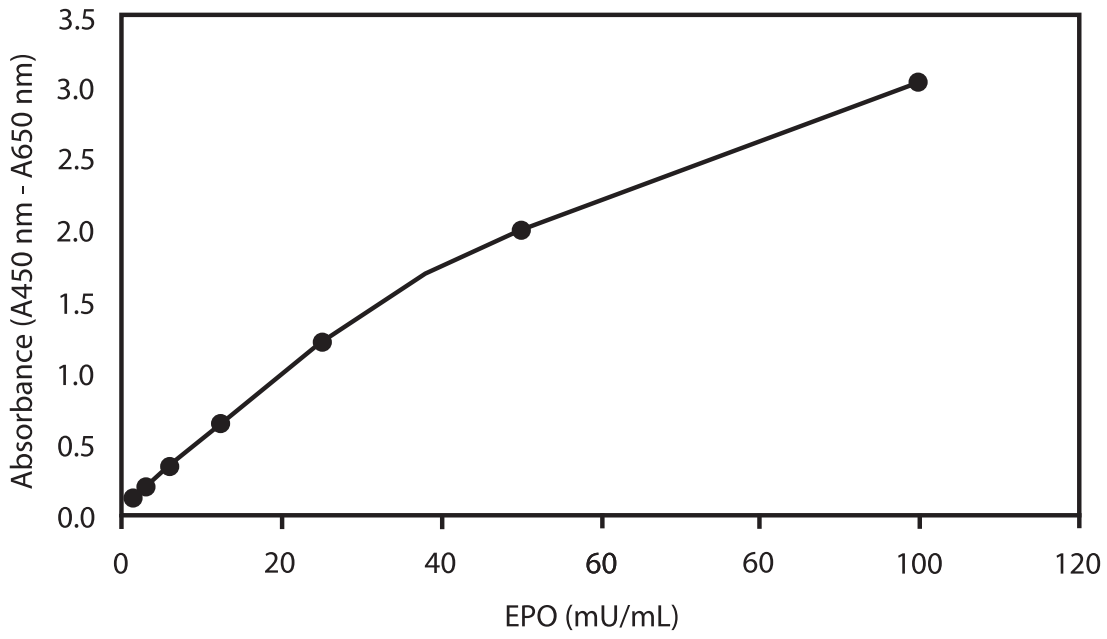
**Please read the entire procedure before beginning the assay.**

1. Prepare 1X Wash Buffer from the 10X Wash Buffer. Add 100 mL of the 10X Wash Buffer to 900 mL of distilled or deionized water. Mix thoroughly and store at 2 - 8°C until use.
2. Bring all reagents to 15 - 25°C, except the TMB Substrate Solution, which can be kept at 2 - 8°C until use.
3. Determine the number of wells required to perform the assay. Standards, samples, and any controls should be tested in duplicate. Carefully remove Microplate from the pouch. Return any strips that are not required for this assay to the pouch. Reseal and store at 2 - 8°C.
4. Add 25 µL of Buffer A to each well.
5. Add 50 µL of standard, buffer or sample to each well (in duplicate).
6. Add 50 µL of Biotinylated Anti-EPO Antibody to each well.
7. Cover the plate with adhesive cover and incubate at 15 - 25°C for 2 hours on the benchtop or for 1 hour on a microplate shaker (500 rpm).
   *Note: We recommend using a shaker as this will give a stronger signal than two hours of incubation without shaking.*
8. After incubation, wash wells five times with 200 µL of the 1X Wash Buffer (prepared in Step 1). Blot dry after the last wash and proceed immediately to the next step.
   *Note: Improper or insufficient washing at any stage of the assay procedure may lead to erroneous results.*
9. Add 100 µL of Streptavidin-HRP Conjugate to each well. Seal plate with a new adhesive cover and incubate at 15 - 25°C for 15 minutes on a microplate shaker (500 rpm) or for 30 - 60 minutes on the benchtop.
   *Note: We recommend using a shaker as this will give a stronger signal than 30 - 60 minutes of incubation without shaking.*
10. Wash wells five times with 200 µL of the 1X Wash Buffer. Blot dry after the last wash and proceed immediately to the next step.
11. Add 100 µL of the TMB Substrate solution to each well. Incubate 15 minutes at 15 - 25°C on the benchtop. A blue color will appear in the wells that contain EPO.
12. Add 100 µL of Stop Solution to each well. The blue color will turn yellow. The absorbance should be measured within 15 minutes after adding the Stop Solution. Use care when handling the Stop Solution (0.5 N sulphuric acid), as it is corrosive!
13. Measure the light absorbance of each well using a microplate reader with the wavelength set at 450 nm. If possible, use a correction wavelength of ≥ 600 nm to correct for optical imperfections. Blank the reader using ELISA wells containing TMB Substrate Solution and Stop Solution only.
    *Note: Remove any air bubbles before measuring the light absorbance using a small hypodermic needle or a pipette tip.*
14. Calculate the average absorbance of each sample from the duplicate values. Subtract the values of the blank (= 0 mU/mL EPO Standard).
15. Generate an EPO Standard curve by plotting the average corrected absorbance values for the eight EPO Standards on the y-axis against the corresponding EPO concentration (in mU/mL) on the x-axis. The data may either be plotted on a double linear scale or a double logarithmic scale. Construct the best-fit curve through the points by regression analysis. The amount of EPO in each test sample is determined by interpolation from the standard curve. The data analysis may also be performed automatically using appropriate data analysis computer software. Please refer to the manual supplied with such software for how to process the data. If the absorbance value of the 100 mU/mol standard is at the upper detection limit of the microplate reader (offscale), omit highest data point from the EPO Standard dose-response curve and perform the data analysis on the data for the remaining seven EPO Standards.
    *Note: If the absorbance value of a test sample is above that of the 100 mU/mL EPO Standard, or the absorbance value of a test sample is off-scale, the EPO concentration in that sample exceeds the upper limit of the assay. To obtain an accurate value the sample must be diluted in Buffer B and retested along with the standards.*

## EPO Standard Curve *Example*

These data of a titration of the EPO Standards performed in duplicate are shown for demonstration purposes only. The EPO Standards should be assayed and a standard curve generated for each individual assay.

| EPO CONC. (mU/mL) | A450 nm - A650 nm | A450 nm - A650 nm | AVERAGE A450 nm - A650 nm | BLANK SUBTRACTED |
|---|---|---|---|---|
| 100 | 3.051 | 3.022 | 3.037 | 2.997 |
| 50 | 1.962 | 2.072 | 2.017 | 1.977 |
| 25 | 1.233 | 1.241 | 1.237 | 1.197 |
| 12.5 | 0.664 | 0.645 | 0.655 | 0.615 |
| 6.25 | 0.376 | 0.359 | 0.368 | 0.328 |
| 3.12 | 0.208 | 0.208 | 0.208 | 0.168 |
| 1.56 | 0.132 | 0.125 | 0.129 | 0.089 |
| 0 | 0.039 | 0.041 | 0.040 | - |



# Performance Characteristics

## Lower Limit of Detection (Sensitivity)

0.6 mU/mL, as determined by adding two Standard Deviations to the Average Absorbance value of 20 replicates of the Zero Standard and reading the EPO concentration from the standard curve.

## Range

1.6 - 100 mU/mL

## Precision

For assessment of intra-assay variation, three samples of recombinant human EPO were tested in 20 replicates. For assessment of inter-assay variation, three samples of recombinant human EPO were tested in triplicate in seven individual tests:

| EPO (mU/mL) | Intra-Assay Variation (% CV*) | Inter-Assay Variation (% CV*) |
|---|---|---|
| 100 | 1.2 | 3.9 |
| 12.5 | 2.4 | 5.9 |
| 1.6 | 3.0 | 5.2 |

* CV = Coefficient of Variation

## Specificity

No reactivity was observed with the following human recombinant cytokines (all tested at 1 µg/mL):
TNF, IL-1, IL-3, IL-6, G-CSF, GM-CSF, SCF, Tpo.

No reactivity was observed with serum from the following animal species: Cow, goat, horse, mouse, rat, sheep, rabbit.
Reactivity was observed with serum from anemic baboons.

# Troubleshooting

| WHAT IS THE PROBLEM? | POSSIBLE CAUSE | RECOMMENDED ACTION |
|---|---|---|
| Absorbance values too low | Incubation time too short | Incubation on an orbital shaker (500 rpm) is recommended (1 hour for the first incubation step; 15 minutes for the second incubation step). If a shaker is not available, longer incubation times are required: 2 hours for the first incubation step; 30 - 60 minutes for the second incubation step. |
| | Used undiluted 10X Wash Buffer | Dilute 10X Wash Buffer 10-fold with distilled or deionized water as described in Step 1 of the Assay Procedure on page 7. |
| | Wash step omitted before adding Streptavidin-Peroxidase Conjugate | Follow sequence of incubation and wash steps as outlined in the Assay Procedure on page 7. |
| Absorbance values too high | Incubation time too long | Reduce incubation times or omit shaking during Streptavidin-HRP Conjugate incubation. Consider performing the data analysis without using the absorbance value for the 100.0 mU/mL standard if that value is off-scale, as described in Step 15 of the Assay Procedure on page 7. |
| | Temperature too high (> 25°C) | Perform assay at a lower temperature. If that is not possible, reduce incubation times or omit shaking. |
| Low absorbance readings despite good color development in the wells | Incorrect wavelength setting of the ELISA reader | Check that measuring wavelength is set at 450 nm and that the ELISA reader has the correct filter for this wavelength. |
| Poor replicates | Insufficient washing | Ensure that each well is washed 5X with equal amount of 1X Wash Buffer (200 - 300 µL per well). Ensure that multi-channel pipette fills and empties reproducibly. Blot wells dry before proceeding to the next step. |
| | Unequal volumes in wells | Make sure that pipettes function properly. |
| | • Carry-over between wells<br>• Evaporation from wells<br>• Splashing of well contents onto adhesive cover | Always use a new adhesive cover for each incubation. Make sure that each well is sealed tightly. Keep filled plates in horizontal position and handle with caution. Do not set shaker too fast. |
| | Samples not mixed after thawing | Vortex samples after thawing. |

| WHAT IS THE PROBLEM? | POSSIBLE CAUSE | RECOMMENDED ACTION |
|---|---|---|
| Poor replicates (continued) | High lipids or particulate matter in samples | Filter or centrifuge samples to pellet aggregates (full-speed in a microcentrifuge). Lipids may concentrate on the surface after centrifugation and may be removed. |
| | Air bubbles in well during measurement in the ELISA reader | Remove air bubbles using a small hypodermic needle or pipette tip. |
| High background | Buffer A was not added at start of procedure | Adhere to the Assay procedure on page 7 (Step 4). Buffer A is essential to prevent non-specific binding of reagents. |
| | Insufficient washing | Ensure that each well is washed 5X with equal amount of 1X Wash Buffer (200 - 300 µL per well). Blot wells dry before proceeding to the next step. |
| No signal with EPO Standards | • Reagents not added in correct sequence<br>• Streptavidin-Peroxidase Conjugate added at same time as other reagents | Follow sequence of incubation and wash steps as outlined in the Assay Procedure on page 7. |
| Inconsistent dose-response curve | Incubation conditions for individual wells not identical | Avoid delays during filling of assay wells; consider reducing pipetting time by pipetting standards and samples from a separate microtiter plate using a multi-channel pipette. Make sure that all reagents are at 15 - 25°C prior to performing the assay. |
| Absorbance values of dilutions of a sample decrease with increasing concentration (prozone or hook-effect) | EPO concentration of the samples exceeds the upper limit of the assay | Dilute the samples and retest along with the EPO Standards. |

## Grid for Recording Assay

Please record samples/standards assayed in the appropriate box below.

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 | A2 | A3 | A4 | A5 | A6 | A7 | A8 | A9 | A10 | A11 | A12 |
| B1 | B2 | B3 | B4 | B5 | B6 | B7 | B8 | B9 | B10 | B11 | B12 |
| C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 |
| D1 | D2 | D3 | D4 | D5 | D6 | D7 | D8 | D9 | D10 | D11 | D12 |
| E1 | E2 | E3 | E4 | E5 | E6 | E7 | E8 | E9 | E10 | E11 | E12 |
| F1 | F2 | F3 | F4 | F5 | F6 | F7 | F8 | F9 | F10 | F11 | F12 |
| G1 | G2 | G3 | G4 | G5 | G6 | G7 | G8 | G9 | G10 | G11 | G12 |
| H1 | H2 | H3 | H4 | H5 | H6 | H7 | H8 | H9 | H10 | H1 | H12 |

## References


1. Papayannopoulou T & Abkowitz J. (1995) Biology of Erythropoiesis, Differentiation, and Maturation. In: Hoffman R, Benz Jr. EJ, Shattil SJ, Furie B, Cohen HJ, Silberstein LE (Eds), Hematology: Basic Principles and Practice, 2nd edition (pp242 - 254). New York: Churchill Livingstone Inc.
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## Notes

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