

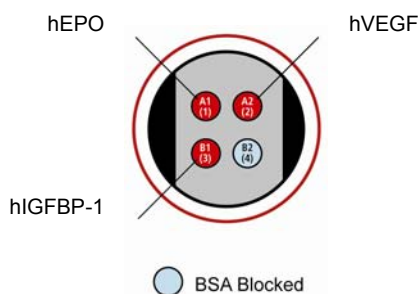
MSD[®] 96-Well MULTI-SPOT[®] Human Hypoxia Tissue Culture Assay

The following assay protocol has been optimized for analysis of human erythropoietin (EPO), insulin-like growth factor binding protein-1 (IGFBP-1) and vascular endothelial growth factor (VEGF) in tissue culture samples.

Storage

MSD Materials

<input type="checkbox"/> Read Buffer T (4X), with surfactant	RT
<input type="checkbox"/> Blocker A Kit	RT
<input type="checkbox"/> MULTI-SPOT 96-well 4 Spot Human Hypoxia Plate(s)	2-8 °C
<input type="checkbox"/> SULFO-TAG [™] Anti-hEPO Antibody (100X) ¹	2-8 °C
<input type="checkbox"/> SULFO-TAG Anti-hIGFBP-1 Antibody (100X) ¹	2-8 °C
<input type="checkbox"/> SULFO-TAG Anti-hVEGF Antibody (100X) ¹	2-8 °C
<input type="checkbox"/> Diluent 1	2-8 °C
<input type="checkbox"/> Diluent 100	2-8 °C
<input type="checkbox"/> Human VEGF Calibrator (1 µg/mL)	≤-70 °C
<input type="checkbox"/> Human EPO Calibrator (20 IU/mL)	≤-70 °C
<input type="checkbox"/> Human IGFBP-1 Calibrator (1 µg/mL)	≤-70 °C



The SECTOR[®] Imager data file will identify spots according to their well location, not by the coated capture antibody name.

¹ Some SULFO-TAG labeled detection antibodies may be light-sensitive, so they should be stored in the dark.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC OR THERAPEUTIC PROCEDURES.



Other Materials & Equipment (not supplied)

- ❑ Deionized water for diluting Wash Buffer and Read Buffer.
- ❑ Phosphate buffered saline with 0.05% Tween-20 (PBS-T) for plate washing
- ❑ Adhesive plate seals
- ❑ Microtiter plate shaker
- ❑ Automatic plate washer or other efficient multi-channel pipetting equipment for washing 96-well plates
- ❑ Appropriate liquid handling equipment for desired throughput that must accurately dispense 25, 50, and 150 μL into a 96-well microplate

Notes:

Read the entire thorough instructions before beginning work.

Protocol at a Glance

The following protocol describes a preferred assay format. The protocol can be completed in approximately 4 hours if each reagent is prepared during the preceding incubation. This time can be reduced to 2.5 hours if the blocking reagent is added the night before.

- Step 1.** Add Blocking Solution, incubate 1 hour, wash. (alternatively, block plates overnight at 4 °C).
- Step 2.** Add 25 μL of Detection Antibody.
Add 25 μL of Samples or Calibrator, incubate 2 hours, wash.
- Step 3.** Add 150 μL of Read Buffer, read plate and analyze data.

Preparation Instructions

Prepare Blocker A Kit:

Prepare Blocker A solution following the instructions included in the Blocker A kit.

Prepare Calibrator dilutions:

1. Determine how many Calibrator levels and replicates will be run. Each well will require 25 μL of Calibrator. Thaw one vial each of EPO, IGFBP-1 and VEGF Calibrator stock solutions and prepare the required Calibrator dilution series using the stock solutions and Diluent 1.
 - A recommended Calibrator dilution procedure is listed below for up to 4 replicates of 7 Calibrator concentrations spanning a wide range, plus 1 zero-Calibrator point.



- Prepare 200 μL of a high combined Calibrator containing 100 ng/mL IGFBP-1, 25 ng/mL VEGF, and 10 IU/mL of EPO by combining 20 μL of IGFBP-1 stock solution at 1 $\mu\text{g}/\text{mL}$, 5 μL of VEGF stock solution at 1 $\mu\text{g}/\text{mL}$, and 100 μL of the 20 IU/mL EPO stock solution with 75 μL of Diluent 1.
 - Prepare 6 additional 1:4 serial dilutions, beginning with the high combined Calibrator, by adding 50 μL of the Calibrator to 150 μL Diluent 1.
 - This will create 7 Calibrators with 100000, 25000, 6250, 1563, 391, 98, 24 pg/mL of IGFBP-1, 25000, 6250, 1563, 391, 98, 24, 6 pg/mL of VEGF, and 10000, 2500, 625, 156, 39, 9.8, 2.4 mIU/mL EPO.
 - The recommended 8th dilution is Diluent 1 alone (e.g. zero Calibrator).
 - ❖ Once the expected range of sample concentrations is known, the Calibrator concentrations can be adjusted appropriately. NOTE: At very high VEGF levels (greater than 25000 pg/mL), the calibration curve may hook. It is recommended that samples with VEGF levels in this range be diluted so that they are measured in the linear portion of the calibration curve.
2. Calibrators are stable at room temperature for a few hours.
 3. The human hypoxia calibrators have been anchored and referenced to international standards when available. The table below summarizes the reference information.

Analyte	WHO Standard Reference Number	WHO Standard Units / μg	MSD Calibrator 1 μg = WHO Units	WHO Units
h EPO	88/574	127	130	IU
h VEGF	01/424	n/a	0.5	μg
h VEGF	02/286	1000	360	U
h IGFBP-1	n/a	n/a	n/a	n/a

** MSD VEGF Calibrator previously used in Human VEGF Kits and Human Hypoxia Kits was anchored to WHO Standard Reference 01/424 with 1 μg of MSD Calibrator = 1 μg of WHO Standard

Prepare Detection Antibody Reagent:

1. Each well will require 25 μL of Detection Antibody Reagent. Prepare 3 mL per plate.
2. In a 15 mL tube combine:
 - a. 2.932 mL Diluent 100
 - b. 30 μL of 100X SULFO-TAG Anti-hEPO Antibody (final concentration: 1X)
 - c. 30 μL of 100X SULFO-TAG Anti-hIGFBP-1 Antibody (final concentration: 1X)
 - d. 7.5 μL of 100X SULFO-TAG Anti-hVEGF Antibody (final concentration: 0.25X)



Notes:

3. Detection Antibody Reagent is stable at room temperature for a few hours.

Prepare Diluted Read Buffer:

- In a 50 mL tube combine (per plate):
- a. 5 mL 4X Read Buffer T
 - b. 15 mL deionized water

Diluted Read Buffer may be kept in a tightly sealed container at room temperature for later use.

Assay Protocol

Begin with a MULTI-SPOT 96-well 4 Spot Human Hypoxia plate. No pre-treatment is necessary.

1. Add 150 μ L/well of blocking solution A and incubate at room temperature for 1 hour or overnight at 4 °C.
2. Wash plates 3 times with phosphate buffered saline + 0.05% Tween-20 (PBS-T).
3. Dispense 25 μ L/well of Detection Antibody Reagent and 25 μ L/well Calibrator, or sample, and incubate at room temperature with shaking for 2 hours.
4. Wash plates 3 times with PBS-T.
5. Prepare SECTOR Imager such that plate can be read immediately after Read Buffer addition.
6. Add 150 μ L/well 1X Read Buffer T.
7. Analyze immediately with SECTOR Imager.

Plates may also be blocked overnight at 4°C and stored for up to a week with blocker.

Shaking a 96-well MSD MULTI-ARRAY[®] or MULTI-SPOT plate typically accelerates capture at the working electrode.

Bubbles in the Read Buffer will interfere with reliable imaging of the plate.

