

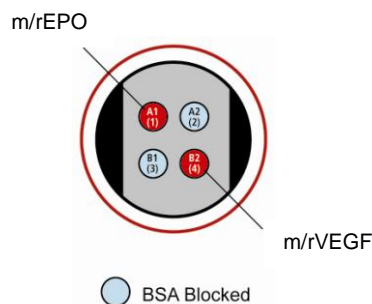
# MSD<sup>®</sup> MULTI-SPOT<sup>®</sup> Assay: Mouse/Rat Hypoxia Panel

The following assay protocol has been optimized for analysis of mouse/rat erythropoietin (EPO) and vascular endothelial growth factor (VEGF) in Serum and Plasma samples.

Storage

## MSD Materials Included

|  |         |
|--|---------|
| <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 m/r Hypoxia Plate(s)                  | 2-8 °C  |
| <input type="checkbox"/> SULFO-TAG <sup>™</sup> Anti-m/rEPO Antibody (100X) <sup>1</sup> | 2-8 °C  |
| <input type="checkbox"/> SULFO-TAG Anti-m/rVEGF Antibody (100X) <sup>1</sup>             | 2-8 °C  |
| <input type="checkbox"/> Diluent 6   | ≤-10 °C |
| <input type="checkbox"/> Diluent 8   | ≤-10 °C |
| <input type="checkbox"/> Diluent 16  | ≤-10 °C |
| <input type="checkbox"/> Mouse VEGF Calibrator (0.1 µg/mL)                               | ≤-70 °C |
| <input type="checkbox"/> Mouse EPO Calibrator (0.1 µg/mL)                                | ≤-70 °C |
| <input type="checkbox"/> Rat VEGF Calibrator (0.1 µg/mL)                                 | ≤-70 °C |
| <input type="checkbox"/> Rat EPO Calibrator (0.1 µg/mL)                                  | ≤-70 °C |



The SECTOR<sup>®</sup> Imager data file will identify spots according to their well location, not by the coated capture antibody name.

<sup>1</sup> 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 + 0.05% Tween-20 (PBS-T) for plate washing
- ❑ Adhesive plate seals
- ❑ Microtiter plate shaker
- ❑ Plate washer or other efficient multi-channel pipetting equipment for washing 96-well plates
- ❑ Liquid handling equipment for desired throughput that must accurately dispense 25, 50, and 150  $\mu\text{L}$  into a 96-well micro plate

*Read the entire detailed instructions before beginning work.*

## ***Protocol at a Glance***

The following protocol describes a preferred assay format. The protocol can be completed in approximately 5.5 hours if each reagent is prepared during the preceding incubation. This time can be reduced to 4.5 hours if the blocking reagent is added the night before.

- Step 1.** Add Blocking Solution, incubate 1-2 hours, wash.  
(alternatively, block plates overnight at 4 °C).
- Step 2.** Add 25  $\mu\text{L}$  of Diluent 16.  
Add 25  $\mu\text{L}$  of Samples or Calibrator, incubate 2 hours, wash.
- Step 3.** Add 25  $\mu\text{L}$  of Detection Antibody, incubate 2 hours, wash.
- Step 4.** Add 150  $\mu\text{L}$  of Read Buffer, read plate and analyze data.

## ***Preparation Instructions***

### **Prepare Blocker A Kit:**

Follow instructions included with the Blocker A kit.

### **Prepare Calibrator dilutions:**

**Depending on the desired application, the following procedure can be applied to Mouse or Rat Calibrators.**

1. Determine the number of Calibrator concentrations and replicates that will be tested. Each well will require 25  $\mu\text{L}$  of Calibrator. Thaw one vial each of EPO and VEGF Calibrator stock solutions and prepare the required Calibrator dilution series using the Calibrator stock solutions and Diluent 6. A sample plate layout is shown in Figure 1.
2.
  - a) A recommended Calibrator dilution procedure is listed below for 3 replicates of 7 Calibrator concentrations, plus 1 zero-Calibrator point.



- Prepare 200  $\mu\text{L}$  of a combined Calibrator containing 10 ng/mL VEGF and 10 ng/mL of EPO by adding 20  $\mu\text{L}$  each of the individual Calibrator stock solutions containing 100 ng/mL of EPO and VEGF, respectively, to 160  $\mu\text{L}$  of Diluent 6.
  - Prepare 200  $\mu\text{L}$  of a 2500 pg/mL Calibrator by adding 50  $\mu\text{L}$  of the combined Calibrator at 10 ng/ml to 150  $\mu\text{L}$  of Diluent 6 (1:4 dilution).
  - Prepare 5 additional 1:4 serial dilutions, by adding 50  $\mu\text{L}$  to 150  $\mu\text{L}$  of Diluent 6.
  - This will create 7 Calibrators containing 10,000, 2,500, 625, 156, 39, 9.8, & 2.4 pg/mL of VEGF and EPO.
  - The recommended 8th Calibrator is Diluent 6 (e.g. zero Calibrator).
- b) Once the expected range of sample concentrations is known, the Calibrator concentrations can be adjusted appropriately.
3. Calibrators are stable at room temperature for a few hours.

**Prepare Detection Antibody Reagent:**

1. Each well will require 25  $\mu\text{L}$  of Detection Antibody Reagent. Prepare 3 mL per plate.
2. In a 15 mL tube combine:
  - a. 2.94 mL Diluent 8
  - b. 30  $\mu\text{L}$  of 100X SULFO-TAG Anti-m/rEPO Antibody (final concentration: 1X)
  - c. 30  $\mu\text{L}$  of 100X SULFO-TAG Anti-m/rVEGF Antibody (final concentration: 1X)
3. Detection Antibody Reagent is stable at room temperature for a few hours.

**Prepare Diluted Read Buffer:**

1. Determine total number of wells in experiment. Each well will receive 150  $\mu\text{L}$  of 1X Read Buffer T, with surfactant.
2. Dilute 4X Read Buffer T, with surfactant to 1X with deionized water.
3. Diluted Read Buffer may be stored at room temperature for later use.



**Notes:**

## Assay Protocol

Begin with a MULTI-SPOT 96-well 4 Spot m/r Hypoxia Plate.  
No pre-treatment is necessary.

1. Add 150  $\mu$ L/well of blocking solution and incubate at room temperature for 1 hour or overnight at 4  $^{\circ}$ C.
2. Wash plates 3 times with phosphate buffered saline + 0.05% Tween-20 (PBS-T).
3. Dispense 25  $\mu$ L/well of Diluent 16 into each well.
4. Dispense 25  $\mu$ L/well of Calibrator or sample, and incubate at room temperature with shaking for 2 hours.
5. Wash plates 3 times with PBS-T.
6. Dispense 25  $\mu$ L/well of Detection Antibody Reagent and incubate at room temperature with shaking for 2 hours.
7. Wash plates 3 times with PBS-T.
8. Prepare SECTOR Imager such that plate can be read immediately after Read Buffer addition.
9. Add 150  $\mu$ L/well 1X Read Buffer T.
10. Analyze immediately with SECTOR Imager. This is important because the EPO assay signal decreases about 15 % over 5 minutes.

*Plates may also be blocked overnight at 4 $^{\circ}$ C and stored for up to a week with blocker.*

*Shaking a 96-well MSD MULTI-ARRAY<sup>®</sup> or MULTI-SPOT plate typically accelerates capture at the working electrode*

*Bubbles introduced during the Read Buffer addition will interfere with reliable imaging of the plate.*

*Changes in EPO assay signal with time in Read Buffer can also be minimized by waiting 10 minutes between Read Buffer T addition and analysis of the plate with the SECTOR Imager.*

|                                   |   | 1                                | 2 | 3 | 4       | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|-----------------------------------|---|----------------------------------|---|---|---------|---|---|---|---|---|----|----|----|
| Calibration curve dilution series | A | Cal7                             |   |   |         |   |   |   |   |   |    |    |    |
|                                   | B | Cal6                             |   |   |         |   |   |   |   |   |    |    |    |
|                                   | C | Cal5                             |   |   |         |   |   |   |   |   |    |    |    |
|                                   | D | Cal4                             |   |   |         |   |   |   |   |   |    |    |    |
|                                   | E | Cal3                             |   |   |         |   |   |   |   |   |    |    |    |
|                                   | F | Cal2                             |   |   |         |   |   |   |   |   |    |    |    |
|                                   | G | Cal1                             |   |   |         |   |   |   |   |   |    |    |    |
|                                   | H | 0                                |   |   |         |   |   |   |   |   |    |    |    |
|                                   |   | blended rat or mouse calibrators |   |   | samples |   |   |   |   |   |    |    |    |

**Figure 1.** Sample plate layout that can be used for this assay.

