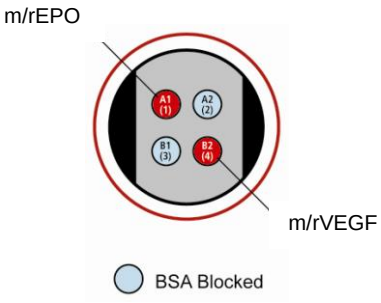
MSD® MULTI-SPOT® Assay: Mouse/Rat Hypoxia Panel for Tissue Culture

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

MSD Materials

	Storage
❑ Read Buffer T (4X), with surfactant	RT
❑ Blocker A Kit	RT
❑ MULTI-SPOT 96-well 4 Spot m/r Hypoxia Plate(s)	2-8 ºC
❑ SULFO-TAG™ Anti-m/rEPO Antibody (100X)[1]	2-8 ºC
❑ SULFO-TAG Anti-m/rVEGF Antibody (100X)[1]	2-8 ºC
❑ Diluent 1	2-8 ºC
❑ Diluent 8	≤-10 °C
❑ Mouse VEGF Calibrator (0.1 µg/mL)	≤-70 °C
❑ Mouse EPO Calibrator (0.1 µg/mL)	≤-70 °C
❑ Rat VEGF Calibrator (0.1 µg/mL)	≤-70 °C
❑ Rat EPO Calibrator (0.1 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.

Notes:

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 µL 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 ^{o}C).

Step 2. Add 25 µL of Samples or Calibrator, incubate 2 hours, wash.

Step 3. Add 25 µL of Detection Antibody, incubate 2 hours, wash.

Step 4. Add 150 µL 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 µL 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 1. A sample plate layout is shown in Figure 1.

a) A recommended Calibrator dilution procedure is listed below for 3 replicates of 7 Calibrator concentrations, plus 1 zero-Calibrator point.
   - *Prepare 200 µL of a combined Calibrator containing 10 ng/mL VEGF and 10 ng/mL of EPO by adding 20 µL each of the individual Calibrator stock solutions containing 100 ng/mL of EPO and VEGF, respectively, to 160µL of Diluent 1 .*
   - *Prepare 200 µL of a 2500 pg/mL Calibrator by adding 50µL of the combined Calibrator at 10 ng/ml to 150 µL of Diluent 1 (1:4 dilution).*
   - *Prepare 5 additional 1:4 serial dilutions by adding 50 µL to 150 µL Diluent 1.*
   - *This will create 7 Calibrators containing 10000, 2500, 625, 156, 39, 9.8, & 2.4 pg/mL of VEGF and EPO.*
   - *The recommended 8th Calibrator is Diluent 1 (e.g. zero Calibrator).*

b) Once the expected range of sample concentrations is known, the Calibrator concentrations can be adjusted appropriately.

2. Calibrators are stable at room temperature for a few hours.

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. 2.94 mL Diluent 8
   b. 30 µL of 100X SULFO-TAG Anti-m/rEPO Antibody (final concentration: 1X)
   c. 30 µL 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 µL 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.

Assay Protocol

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

1. Add 150 µL/well of blocking solution 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 µL/well of Calibrator or sample, and incubate at room temperature with shaking for 2 hours.
4. Wash plates 3 times with PBS-T.
5. Dispense 25 µL/well of Detection Antibody Reagent and incubate at room temperature with shaking for 2 hours.
6. Wash plates 3 times with PBS-T.
7. Prepare SECTOR Imager such that plate can be read immediately after Read Buffer addition.
8. Add 150 µL/well 1X Read Buffer T.
9. Analyze immediately with SECTOR Imager. This is important because the EPO assay signal decreases about 20% over 5 minutes.

Notes:

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

Shaking a 96-well MULTI-ARRAY® 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.

Additional notes:

1) Alternative protocols can be used to shorten the total assay time with a compromise in assay sensitivity or performance. These alternative methods can be evaluated depending on the specific application.
Options include:

1. simultaneous incubation of detection antibody and sample or Calibrator
2. eliminating the block step followed by simultaneous incubation of Detection Antibody and sample or Calibrator
3. simultaneous incubation of Detection Antibody and sample or Calibrator followed by eliminating the wash step at the end of the assay (add MSD Read Buffer T at a final concentration of 1X immediately prior to reading the plate)

2) In situations where the VEGF signal is too high as compared to the EPO signal, the following protocol modification allows the reduction of VEGF signal. The SULFO-TAG VEGF Antibody should be diluted to a final concentration of 0.3X and unlabeled VEGF Antibody (provided upon request) should be added to the Detection Antibody Reagent. Contact MSD technical support (CustomerSupport@mesoscale.com) for more information on this method.