

# Detection of $O_2^{\cdot-}$ production by chemiluminescence (CL) assay

**CURRENT STATUS:** POSTED

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**DOI:**

10.1038/nprot.2007.485

**SUBJECT AREAS**

*Biochemistry*    *Computational biology and bioinformatics*

**KEYWORDS**

*superoxide ion, chemiluminescence, Cypridina luciferin analog*

## Introduction

Hemoglobin is auto-oxidized from ferrous-hemoglobin to ferric hemoglobin (methemoglobin, metHb) with the production of  $H_2O_2$ .  $H_2O_2$  initiates the catalytic cycle between the ferric (HbFeIII) and ferryl (HbFeIV) hemes, thus activating the pseudoperoxidase activity of metHb, which eliminates the  $H_2O_2$ , producing the superoxide<sup>1</sup>. The chemiluminescence (CL) of Cypridina luciferin analog indicates the generation of  $O_2^{\bullet-}$  or singlet oxygen, but not that of ozone, hydroxyl radicals or  $H_2O_2$ <sup>2</sup>. To further confirm that the ROS species produced by metHb and indicated by CLA-CL is superoxide anions but not singlet oxygen, superoxide dismutase (SOD) is applied as a diagnostic confirmation of superoxide anion production.

## Procedure

The pseudoperoxidase activity is measured as described <sup>2</sup>, with modifications.

1. Add metHb into a substrate mixture containing 5.3  $\mu$ M of CLA and 3.3 mM of  $H_2O_2$  in 150  $\mu$ l of PBS (pH 7.4).
2. Continuously monitor the chemiluminescence, indicated by relative luminescence units (rlu), at one reading per s, for 1 min, with Sirius luminometer (Berthold Detection Systems GmbH, Germany).
3. The pseudoperoxidase activity of the metHb is designated as the relative luminescence units per second (rlu/s).
4. To prove that the ROS species indicated by CLA-CL is superoxide anions, 3 Units/ml of SOD is applied to specifically quench the superoxide anions.

## Anticipated Results

The read out of CLA-LA varies from  $10^5$ - $10^7$  rlu/s depending on the concentration of the metHb and the  $H_2O_2$  as the substrate as well as that of CLA as the indicator.

## References

1. Alayash, A. I. Hemoglobin-based blood substitutes: oxygen carriers, pressor agents, or oxidants? *Nat Biotechnol.* **17**, 545-549 (1999).

2. Kawano, T., Pinontoan, R., Hosoya, H. & Muto, S. Monoamine-dependent production of reactive oxygen species catalyzed by pseudoperoxidase activity of human hemoglobin. *Biosci Biotechnol Biochem.* **66**, 1224-1232 (2002).

## Figures

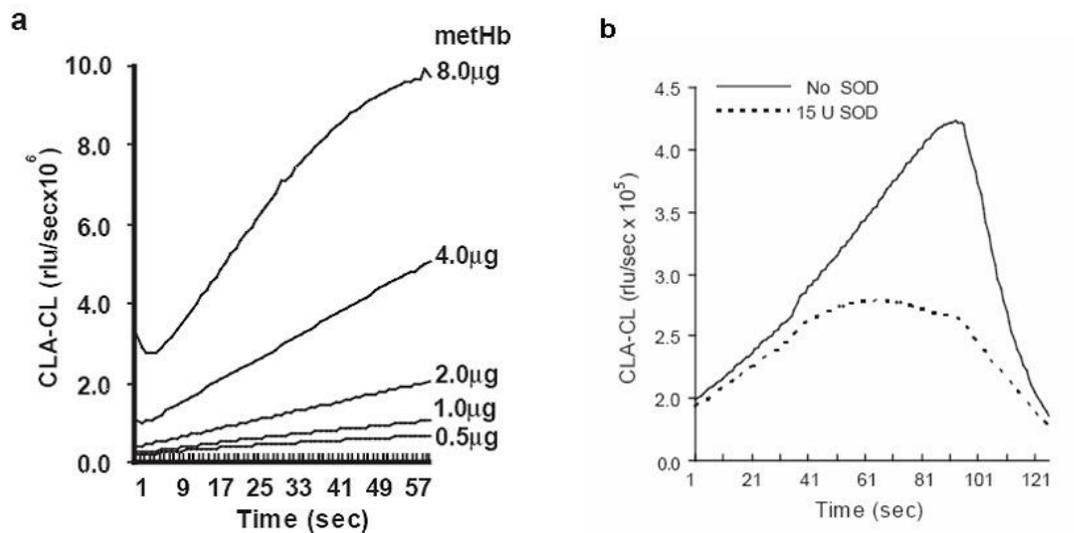


Figure 1

The pseudoperoxidase activity of methemoglobin (methHb) was demonstrated by CLA-CL. (a)

methHb catalyzes the production of  $O_2^{\cdot -}$ , as shown by the

chemiluminescence (CL) assay. (b) SOD significantly reduces methHb-mediated CLA-

luminescence, thereby confirming the superoxide anion.

## Respiratory protein-generated reactive oxygen species as an antimicrobial strategy

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