Exposing and detecting membrane and intracellular proteins: AlphaLISA simplifies the detection of HO-1.

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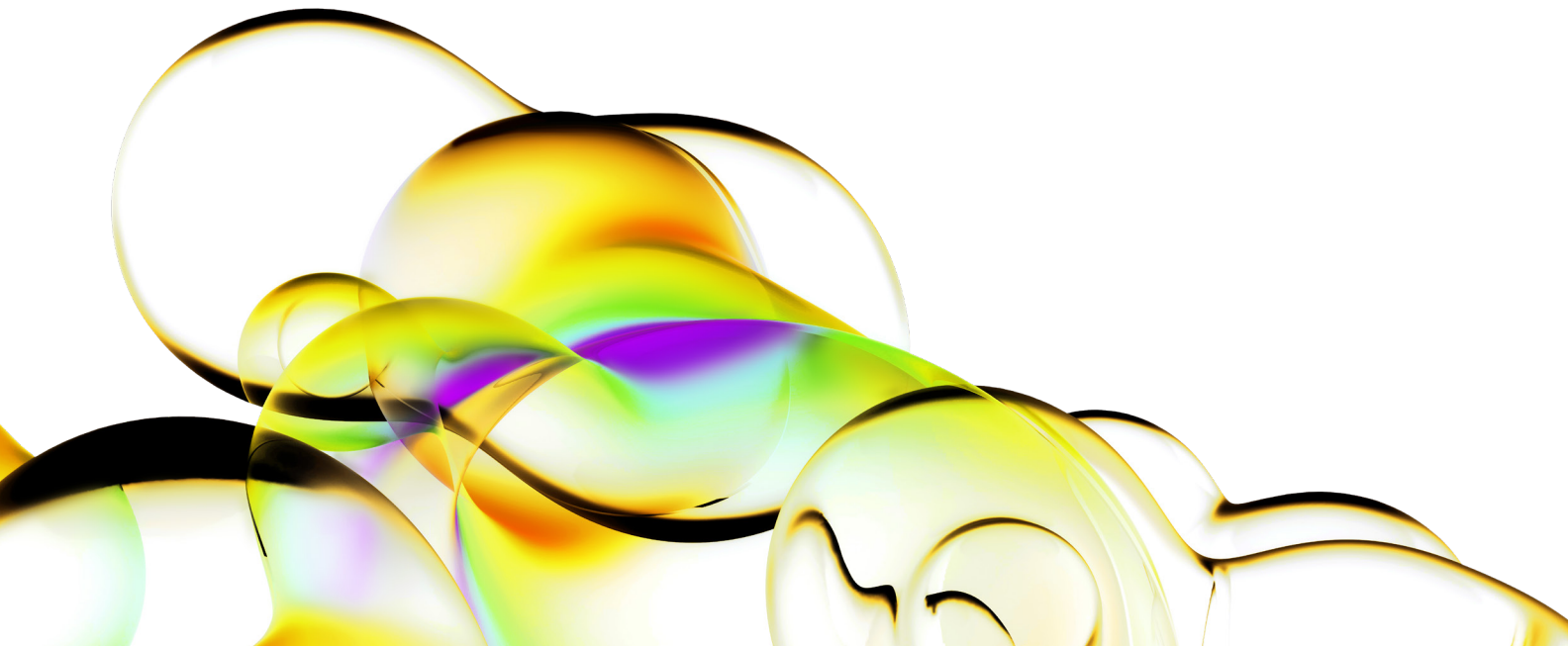
Introduction

AlphaLISA™ technology is demonstrated to greatly simplify the detection of the endoplasmic reticulum associated protein heme oxygenase 1 (HO-1), reducing a complex procedure to extract and expose HO-1 to a one lysis step, mix and read assay. HO-1 and other membrane associated proteins that are either anchored or have a single trans-membrane domain are often irreversibly denatured by attempts to extract them from the membrane unless special precautions are taken. Here we present a simplified procedure and assay based on Revvity's AlphaLISA technology to detect HO-1. Heme oxygenase is a ubiquitously expressed enzyme that is known to play a critical role in protecting cells from oxidative and chemical stress. Metabolic diseases such as insulin resistance, type II diabetes and obesity are associated with low level, chronic inflammation, oxidative stress and endoplasmic reticulum stress.[1] HO-1 has been shown to play an important role in the pathogenesis of metabolic disease through its role in reducing inflammatory, oxidative and ER stresses by exerting antioxidant, anti-inflammatory and antiapoptotic effects and therefore is becoming an attractive therapeutic target.[2,3]

Here, we demonstrate how AlphaLISA technology can be used to quantify levels of HO-1 in HeLa cell extracts. Combining our AlphaLISA no wash technology with AlphaLISA lysis buffer, we have shown that it is possible to liberate the ER associated protein, HO-1, in one step instead of using the typical protocols to destroy both the cell membrane and the ER membrane to liberate HO-1 through complex, multistep cell fractionation.



AlphaLISA technology allows for the detection of molecules of interest in buffer, cell culture media, serum, plasma and cell lysates and in a highly sensitive, quantitative, reproducible and user-friendly mode. In an AlphaLISA assay, a biotinylated anti-analyte antibody binds to the Streptavidin-coated Alpha Donor beads, while another anti-analyte antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer within the Acceptor beads, resulting in a sharp peak of light emission at 615 nM (Fig. 1). This light emission can then be detected on an Alpha-enabled reader.

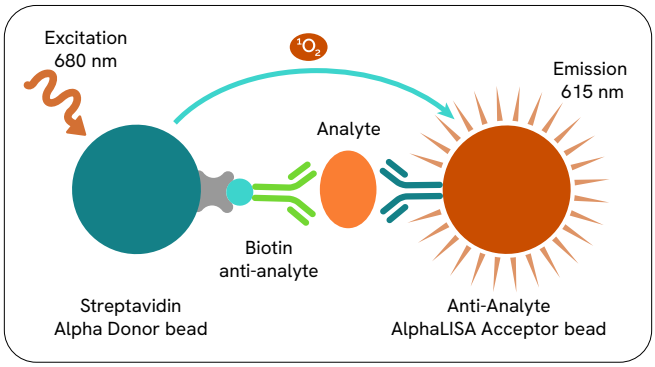


Figure 1: AlphaLISA assay schematic. In the presence of analyte, antibodies sandwich the analyte and bring the Donor beads and Acceptor beads within close proximity. Upon excitation, singlet oxygen from the Donor beads diffuses and activates nearby Acceptor beads to generate light that is proportional to the amount of analyte in the sample of interest.

Materials and methods

HeLa cells

HeLa cells (ATCC® CCL-2™) were maintained in 75 mL cell culture flasks (Corning 430641U) in RPMI 1640 (ThermoFisher 11875-093) supplemented with 10% fetal bovine serum (FBS, ThermoFisher 10010-23). Cells were harvested once they reached confluence by removing cell culture media, washing with 10 mL of PBS, and then detaching the cells with 5 mL of Versene (ThermoFisher 15040-066) for 15 minutes (gentle tapping of the flask was required to detach cells). The cells were then diluted to 10 mL with culture media and counted manually using 0.4% Trypan Blue solution (ThermoFisher 15250-061) and a hemocytometer.

Detection of human HO-1 using AlphaLISA technology

The AlphaLISA immunoassay for human heme oxygenase 1 (Revvity #AL3026 HV/C/F) was performed according to the recommended protocol provided with the kit (Fig. 2).

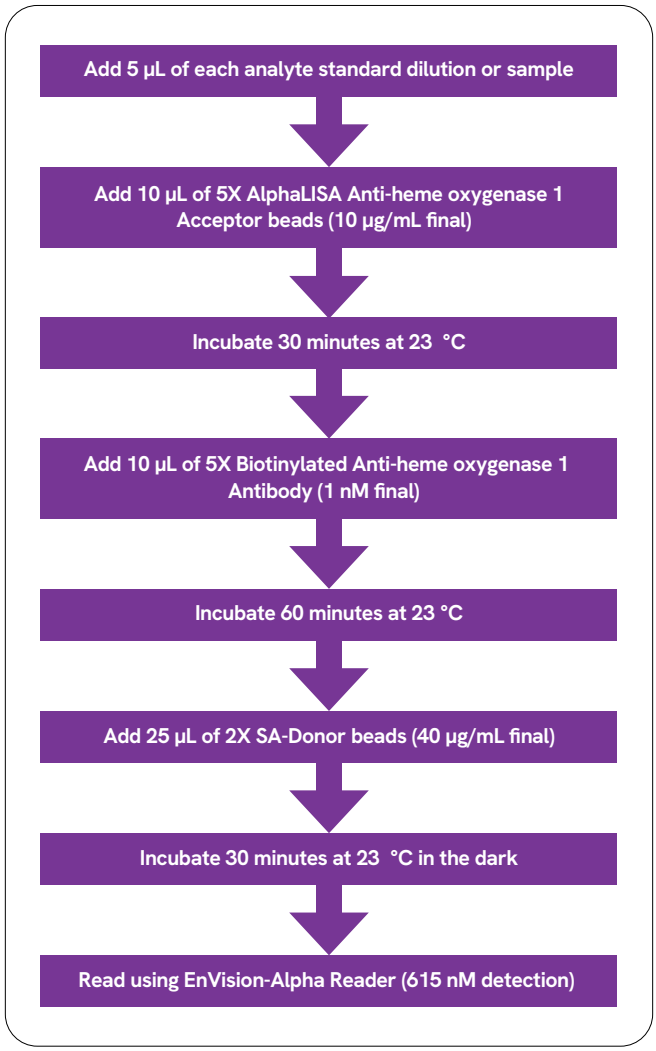


Figure 2: Workflow for human heme oxygenase 1 AlphaLISA detection assay.

Standard curve/sensitivity

Sensitivity of AlphaLISA to HO-1 (Revvity #AL3026) in AlphaLISA Lysis Buffer was measured. The data were generated using a white Optiplate™ 384 microplate and the EnVision™ Multimode Plate Reader 2103 with Alpha option.

HO-1 extraction

In order to assess the presence and concentration of the membrane associated analyte, HO-1, cells were first lysed. To do this, media was removed by aspiration, cells washed twice with sterile PBS and then incubated at room temperature for 30 minutes in 50 uL of 1X lysis buffer (AlphaLISA Lysis Buffer 5X; Revvity #AL003C). A 5 µL sample of the resulting lysate was transferred to a 384-well OptiPlate for analysis.

Instrumentation

All AlphaLISA measurements were performed on the Revvity EnVision™ multimode plate reader (Fig. 3) which provides fast, sensitive Alpha technology detection, in addition to fluorescence intensity, luminescence, absorbance, fluorescence polarization, and time-resolved fluorescence detection technologies. The system is based on hybrid technology, combining filters and a monochromator for enhanced flexibility. It incorporates proprietary Direct Double Optics™ technology for high speed and sensitivity in simultaneous filter-based readouts, such as FRET assays, and for TRF, lamp-based excitation is standard, with the option of a high energy laser for higher speed and sensitivity.

Figure 3: EnVision plate reader.

Data analysis

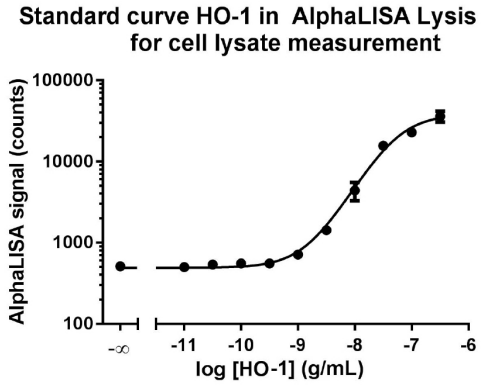
Protein levels in cellular lysates were quantitated by the interpolation of the AlphaLISA signal from the standard curve. Standard curves were prepared using the recombinant standards provided in the AlphaLISA kit. Standard curves were plotted in GraphPad Prism version 6.0 using nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) with $1/Y^2$ weighting method. For spike-and-recovery experiments, percent recovery was calculated by dividing interpolated serum spikes by the associated interpolated diluent spikes then multiplying by 100 to convert to percent. Spike values were determined by comparing to a standard curve prepared in 1X AlphaLISA Lysis Buffer. Additionally, all real sample concentrations were interpolated onto a standard curve prepared in the lysis buffer. Further, lower limit of detection (LDL) was calculated by averaging the blank wells (wells without analyte) and adding three times the standard deviation then interpolating the concentration from the standard curve. Below are the formulas.

$$\text{\% Recovery} = \frac{\text{(spiked sample value/expected sample value) X 100}}{\text{LDL = mean (blanks) + 3 X SD}}$$

Results

Standard curve

Figure 4 shows an AlphaLISA human HO-1 calibration curve performed with 5 µL of HO-1 standard ranging from 10-300,000 pg/mL under optimized assay conditions. The AlphaLISA signal obtained was plotted against the HO-1 concentrations tested.



Kit	LDL (pg/mL)	Dynamic range (pg/mL)	S/B
HO-1	46.5	46.5 - 300,000	102

Figure 4: Typical performance HO-1 AlphaLISA Immunoassays. The standard curve shown was performed in AlphaLISA lysis buffer. The table displays the performance characteristics for the assay.

HO-1 detection in HeLa extracts

Data presented in Table 1 suggest our protocol is sufficient to break the plasma membrane and disrupt endoplasmic reticulum association of HO-1, allowing its release and measurement with AlphaLISA technology. Additionally, proteases potentially released during cell lysis seem to have no negative effect on the AlphaLISA assay. However, this may not be true of all analytes and should be considered when investigating other targets.

Table 1: Heme oxygenase 1 measured by AlphaLISA in HeLa extracts.

Number of cells	Amount of HO-1 (pg/mL)
40,000	30,900
20,000	15,400
10,000	6,630
5,000	2,900
2,500	1,440
1,250	700
625	<LDL

Conclusion

In this technical note, we demonstrated the performance of the HO-1 AlphaLISA detection kit and showed its applicability in lysed HeLa cells. AlphaLISA is a bead-based assay technology that requires no washing and provides excellent assay performance with regard to signal-to-background, dynamic range, and sensitivity. These data demonstrate how Alpha technology can be used to detect intracellular proteins and membrane-associated proteins in a straightforward process. These assays are versatile, easy to perform, sensitive and cost-effective, with few parameters needing to be optimized depending on the target and its location.

References

1. Son, Y., et al. (2013). Therapeutic roles of heme oxygenase-1 in metabolic diseases: curcumin and resveratrol analogues as possible inducers of heme oxygenase-1. *Oxidative Medicine and Cellular Longevity,* 2013: 1-12.

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3. Choi, AM., et al. (1996). Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. *Am J Respir Cell Mol Biol,* 15 (1): 9-19.

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