Automation of an assay to determine hydrogen peroxide levels in THP-1 cells on the OT-2

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INTRODUCTION

- Hydrogen peroxide (H_2O_2) is predominantly associated with cellular damage; however, recent studies show that cells release low levels of H_2O_2 as part of normal intercellular communication. The mechanisms of hydrogen peroxide transport, uptake and release, and biological effects are not yet well known but have important implications for cancer, stem cells, and aging.
- Amplex Red reagent is non-fluorescent
- The assay principle is simple, based on oxidation of 10-acetyl 3,7 dihydroxypenoxazine which is catalyzed by horse radish peroxidase (HRP) in the presence of H_2O_2 to produce a red fluorescent product, resorufin at 1:1 stoichiometry
- Resorufin end-product has a high extinction coefficient
- It is an ultrasensitive assay and as little as 10 picomoles of H_2O_2 in a 100 µL volume; 1×10^{-5} U/mL of HRP
- The readings can be taken spectrophotometrically, or fluorescence readings can be taken using a microplate reader at excitation 530 nm and emission 590 nm.

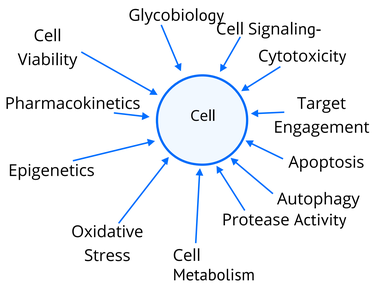


Fig. 1 Schematic representation of cell based assays and the varied cell studies that can be studied

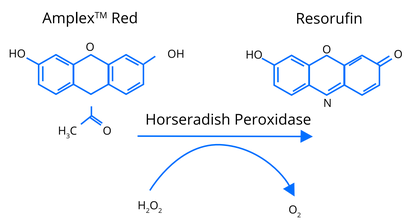


Fig. 2 Amplex Red Assay principle and reaction

MATERIALS AND METHODS

- OT-2, P300 Single GEN2 pipette, P20 Single GEN2 pipette
- Temperature Module set at 23°C and at the end of the protocol at 37°C.
- THP-1 Macrophage-like cells (ATCC, Manassas, VA, USA) (No.TIB-202)
- Phenol red Free RPMI 1640 medium (Thermofisher Scientific 11835030)
- Phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich P1585-1MG)
- Fetal Bovine Serum (FBS) Not Heat Inactivated (ATCC, Cat No. 30-2020)
- Amplex Red assay kit (Cat No. A22188) including a) 5X Reaction Buffer b) HRP c) DMSO d) Amplex Red reagent e) 3% H_2O_2
- Labware a) Opentrons 96 Filter Tip rack 200µL b) Opentrons 96 Filter Tip rack 20µL c) Opentrons 10 Tube Rack with Falcon 4 x 50 mL, 6 x15mL Conical d) Opentrons 24 Tube Rack with Eppendorf 1.5 mL Safe-Lock Snap cap (Two)

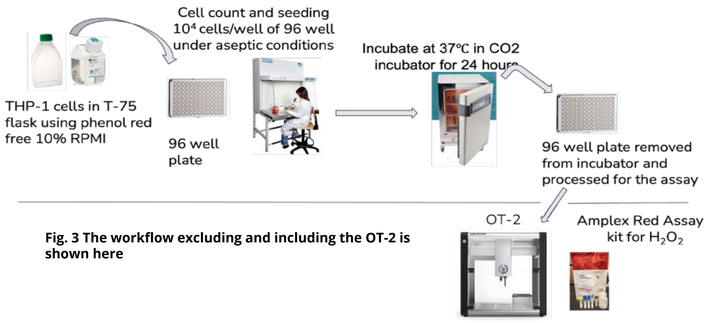
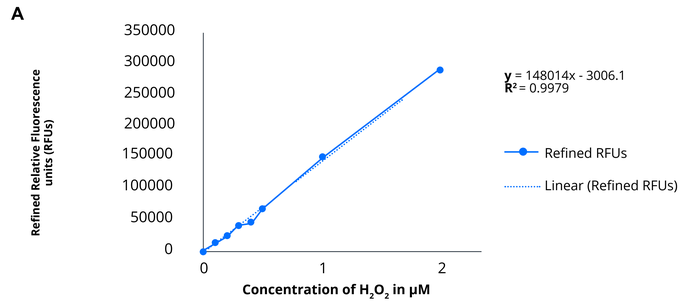


Fig. 3 The workflow excluding and including the OT-2 is shown here

RESULTS



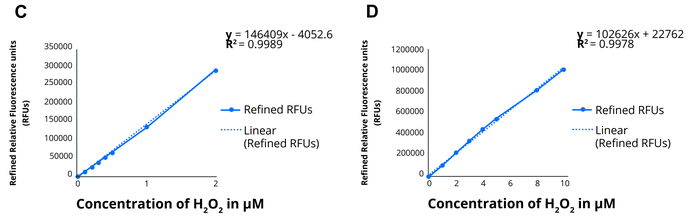


Fig. 4 A & B. Standard curves for H_2O_2 prepared on OT-2. C & D. Standard curves for H_2O_2 prepared manually.

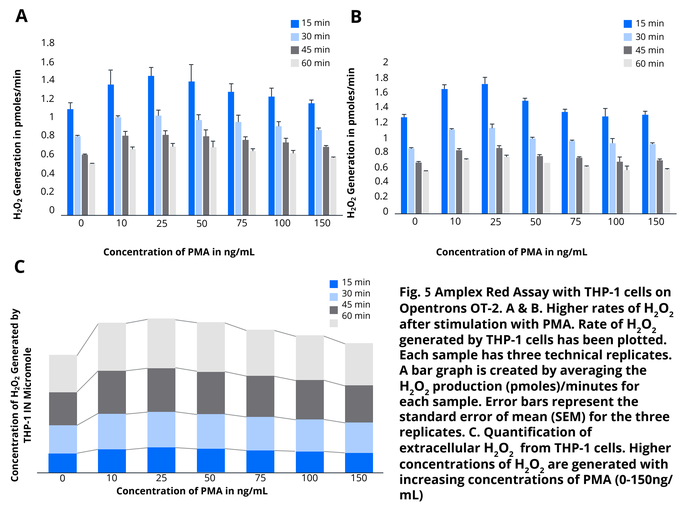


Fig. 5 Amplex Red Assay with THP-1 cells on Opentrons OT-2. A & B. Higher rates of H_2O_2 after stimulation with PMA. Rate of H_2O_2 generated by THP-1 cells has been plotted. Each sample has three technical replicates. A bar graph is created by averaging the H_2O_2 production (pmoles)/minutes for each sample. Error bars represent the standard error of mean (SEM) for the three replicates. C. Quantification of extracellular H_2O_2 from THP-1 cells. Higher concentrations of H_2O_2 are generated with increasing concentrations of PMA (0-150ng/mL)

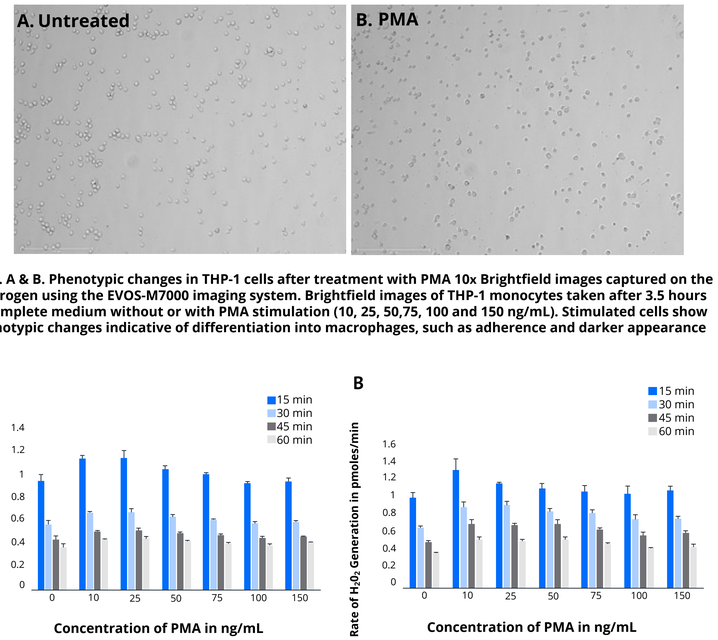


Fig 6. A & B. Phenotypic changes in THP-1 cells after treatment with PMA 10x Brightfield images captured on the Invitrogen using the EVOS-M7000 imaging system. Brightfield images of THP-1 monocytes taken after 3.5 hours in complete medium without or with PMA stimulation (10, 25, 50,75, 100 and 150 ng/mL). Stimulated cells show phenotypic changes indicative of differentiation into macrophages, such as adherence and darker appearance

Fig 7. A & B. Rate of H_2O_2 generated from THP-1 cells showing consistent assay performance after using tilted adaptor for the assay

CONCLUSION

- OT-2 can perform the automation of an assay to determine H_2O_2 in THP-1 cells
- OT-2 can process a moderate to high throughput number of samples in a 96 well format
- OT-2 can mimic the manual protocol without loss of time and highly reproducible

DISCUSSION

This study is carried out to understand feasibility of automating a cellular assay like Amplex Red for the detection of H_2O_2 on the OT-2. H_2O_2 is shown to be an important signaling molecule and is responsible for metabolic activity and aid in enzyme activation, can affect cell morphology, and result in proliferation in the presence of growth factors. Extracellular H_2O_2 is measured in this assay and here the cell line used are human monocytic cells, THP-1. Results indicate possible concentration dependent H_2O_2 production rates among various PMA environments. A relationship between H_2O_2 release rates and PMA concentration is likely following significant increases at 10, 25 ng/mL of PMA over basal rates. The OT-2 has been used successfully to automate various experimental protocols in NGS, IP and thus ease the use of standard kits and assay protocols that can be manually labor intensive. By automating the assay steps, consistent assay performance is observed when compared to the manual protocol . Also, this depicts the comparable pipetting efficiencies between manual and automated protocol that quantify peroxides/peroxidases. This protocol is able to give the user a hands-on time of an hour and eleven minutes and can be an efficient method if multiple 96 well plates with different cell types need to be assayed for hydrogen peroxide levels with or without the addition of various stimulant or growth factors.

REFERENCES

1) Amplex Red Assay Manual from Thermofisher Scientific
2) J G Mohanty , J S Jaffe, E S Schulman, D G Raible A highly sensitive fluorescent micro-assay of H_2O_2 release from activated human leukocytes using a dihydroxyphenoxazine derivative 1997 Mar 28;202(2):133-41. doi: 10.1016/s0022-1759(96)00244-x J Immunol Methods
3) Ozgur Karakuzu, Melissa R. Cruz, Yi Liu, and Danielle A. Garsin, Amplex Red Assay for Measuring Hydrogen Peroxide Production from Caenorhabditis elegans Bio Protocol 2019 Nov 5; 9(21): e3409