# $24,25(OH)_2D_3$ and Regulation of Catalase Activity in LNCaP Prostate Cancer Cells

**Honors Thesis in Biomedicine C15, 15 ECTS**

**(2007-01-17 – 2007-06-08)**

# ABSTRACT

Title: $24,25(OH)_2D_3$ and Regulation of Catalase Activity in LNCaP Prostate Cancer Cells

Department: School of Life Sciences, University of Skövde

Course: Honors Thesis in Biomedicine C15, 15 ECTS

Author: Anette Stahel

Supervisor: Dennis Larsson

Examinator: Karin Klinga Levan

Date: 2007-01-17 – 2007-06-08

Keywords: prostate cancer; proliferation; growth inhibition; vitamin D; $24,25(OH)_2D_3$; catalase; hydrogen peroxide; $H_2O_2$

The vitamin D metabolite $1,25(OH)_2D_3$ has long been known to inhibit growth of prostate cancer cells and this has been attributed to a VDR-mediated pathway controlling target gene expression, resulting in cell cycle arrest, apoptosis and differentiation. New research has shown that another vitamin D metabolite, $24,25(OH)_2D_3$, inhibits proliferation of prostate cancer cells as well, more specifically, cells of the line LNCaP. It is not clear exactly how $24,25(OH)_2D_3$ exerts this cancer growth inhibition but it has been shown that it is to some extent regulated via G protein coupled signalling pathways. Catalase is a haem-containing redox enzyme found in the majority of animal cells, plant cells and aerobic microorganisms. This enzyme is very important because it prevents excessive accumulation of the strongly oxidizing agent $H_2O_2$ which otherwise can do damage to the cells. Because of this preventive effect of catalase, important cellular processes which generate $H_2O_2$ as by-product can proceed safely. Biochemical analysis of catalase has shown that it binds endogenously to $24,25(OH)_2D_3$. The fact that $24,25(OH)_2D_3$ has anti-proliferative effects on prostate cancer cells combined with the fact that it binds to catalase generates the hypothesis that this binding interferes with the essential task of catalase to keep the cell free from accumulation of destructive $H_2O_2$, and by means of this interference induces apoptosis. Finding out about the cancer growth inhibiting mechanism behind each vitamin D metabolite is important and may be a lead in the search for a new, better treatment of prostate cancer. The specific aim of this project was to study if and in what way $24,25(OH)_2D_3$ affects the enzymatic activity of catalase in LNCaP cells and to do this with dose and time responses in focus. In this experiment LNCaP cells were incubated for 48 hours together with $24,25(OH)_2D_3$ in five different concentrations, then a catalase assay was performed on the cells including fluorescence-mediated measuring of catalase activity in both treated and untreated cells. The analysis of the result values showed that regardless of dose or time, $24,25(OH)_2D_3$ has no statistically significant effect on catalase activity in cells of the line LNCaP.

# Table of Contents

# 1 Introduction

## 1.1 Prostate cancer

The prostate is a gland located beneath the urinary bladder in men, surrounding the upper part of the urethra. In this gland a liquid is produced which nourishes the sperm and strengthens them. Both growth and function of the gland are regulated by the male hormone testosterone (Nystrand, 2005).

Cancer of the prostate is the most common form of male cancer. Each year, around half a million cases are diagnosed, worldwide. In Sweden , prostate cancer now accounts for a little more than 35% of all male cancers. The disease is much more common in Western countries than in for instance Asia, and in the USA, black people run a much greater risk of developing the disease than white people. Increasing age is another risk factor for this type of cancer (Nystrand, 2005).

It has been suggested that differences in vitamin D levels account for mentioned observations. Firstly, it is a fact that Japanese men consume larger amounts of fatty fish, the main dietary source of vitamin D, than do Western men (Zhao & Feldman, 2001). Secondly, light skin compared to dark contains less melanin, a compound in the skin which inhibits synthesis of vitamin D. Third, as men age their serum vitamin D levels decrease as the efficiency of vitamin D synthesis decreases with age (Holick, 2005). These suggestions are also supported by newer research showing that vitamin D has anti-proliferative effects on prostate cancer cells (discussed further below).

The symptoms of prostate cancer mainly consist of various urination difficulties and usually do not show until the tumor has spread outside the prostate capsule. The main medical treatments of this disease are prostatectomy, radiation therapy and testosteron ablation. Even though the prognosis of prostate cancer often is good, it is still the form of cancer linked to the highest death rates - in 2002, 2 352 Swedish men died with the disease – and the side-effects of the treatments are rather severe, the most common being impotence, incontinence and hot flushes (Nystrand, 2005). Because of this, more effective treatments with less side-effects are required.

## 1.2 Vitamin D metabolites and prostate cancer

Vitamin D functions like a hormone in the body, and its active form is called $1,25(OH)_2D_3$. The metabolic chain forming $1,25(OH)_2D_3$ begins with photolyzation of 7-dehydrocholesterol by UV light which produces previtamin $D_3$ (Holick *et al.*, 1987). Thereafter, previtamin $D_3$ is hydroxylated at the 25-position in the liver, forming $25(OH)D_3$ (Masumoto *et al.,* 1988), followed by 1-hydroxylation of $25(OH)D_3$ in the kidneys (Lawson *et al.,* 1971). Alternatively a hydroxyl group is added to the side chain of $25(OH)D_3$ or $1,25(OH)_2D_3$, forming $24,25(OH)_2D_3$ or $1,24,25(OH)_2D_3$, respectively. Formation of $24,25(OH)_2D_3$ and $1,24,25(OH)_2D_3$ is believed to be the first inactivation step of the vitamin D metabolites as these products have lower biological activity than does $1,25(OH)_2D_3$ (Akeno *et al.*,1997).

$1,25(OH)_2D_3$ together with parathyroid hormone are major regulators of mineral homeostasis and bone metabolism. $1,25(OH)_2D_3$ aids intestinal calcium absorption and is important for prevention of diseases such as rickets and osteomalacia (Zhao & Feldman, 2001). The cellular receptor for $1,25(OH)_2D_3$ is a nuclear receptor called the Vitamin D Receptor (VDR). The

genes regulated upon binding with the VDR include genes important for calcium metabolism such as osteocalcin, osteopontin, 24-hydroxylase and calbindin (Haussler *et al.,* 1998) but also genes involved in cellular proliferation and differentiation such as c-myc, c-fos, p21, p27 and Hox A10 (Freedman, 1999).

Expressing VDR, the prostate, especially the tumorous prostate (Krill *et al.*, 2001), is a target organ for vitamin D and $1,25(OH)_2D_3$ has long been known to inhibit growth of prostate cancer cells. This has been ascribed to a VDR-mediated pathway controlling target gene expression, resulting in cell cycle arrest, apoptosis and differentiation (Lou *et al.*, 2004).

However, new research has shown that also $24,25(OH)_2D_3$ inhibits proliferation of prostate cancer cells, more specifically, cells of a line called LNCaP (Hagberg, 2006; Sahlberg, 2006). These are cancer cells from the left supraclavicular lymphnode metastasis of a 50-year-old man with prostate carcinoma in 1977 (Horoszewicz, 1981) and they are commonly used in cancer research and drug investigation on which to test the effects of different agents (Winkler *et al.*, 2005). It is not clear exactly how $24,25(OH)_2D_3$ exerts this cancer growth inhibition but it has been shown that it is to some extent regulated via G protein coupled signalling pathways (Björsson, 2006).

### 1.3 The enzyme catalase

Catalase is an enzyme, a haem-containing redox protein, which is found in the majority of animal cells, plant cells and aerobic microorganisms. It is concentrated mainly to the peroxisomes in eurcaryotic cells. This enzyme is very important because it prevents excessive accumulation of the strongly oxidizing agent hydrogen peroxide ($H_2O_2$) which otherwise can do damage to the cells. Because of this preventive effect of catalase, important cellular processes which generate $H_2O_2$ as by-product can proceed safely (Zámocký & Koller, 1999).

Biochemical analysis of catalase has shown that it binds endogenously to the vitamin D metabolites $24,25(OH)_2D_3$, $25(OH)D_3$ and $1,25(OH)_2D_3$. The enzyme binds similarly effective to $24,25(OH)_2D_3$ and $25(OH)_2D_3$, while the binding capacity to $1,25(OH)_2D_3$ is lower (Larsson *et al.*, 2006).

### 1.4 Hypothesis

The fact that vitamin D has anti-proliferative effects on prostate cancer cells combined with the fact that mentioned vitamin D metabolites, particularly $24,25(OH)_2D_3$, bind to catalase generates a hypothesis. That is that this binding interferes with the essential task of catalase to keep the cell free from accumulation of destructive $H_2O_2$, and by means of this interference induces apoptosis.

Finding out about the cancer growth inhibiting mechanism behind each vitamin D metabolite is important and may be a lead in the search for a new, better treatment of prostate cancer. The time has now come to find out about the mechanism behind the action of $24,25(OH)_2D_3$.

# 2 Aim of this Honors Thesis

The specific aim of this project was to study if and in what way $24,25(OH)_2D_3$ affects the enzymatic activity of catalase in LNCaP cells, and to do this with dose and time responses in focus.

# 3 Materials and methods

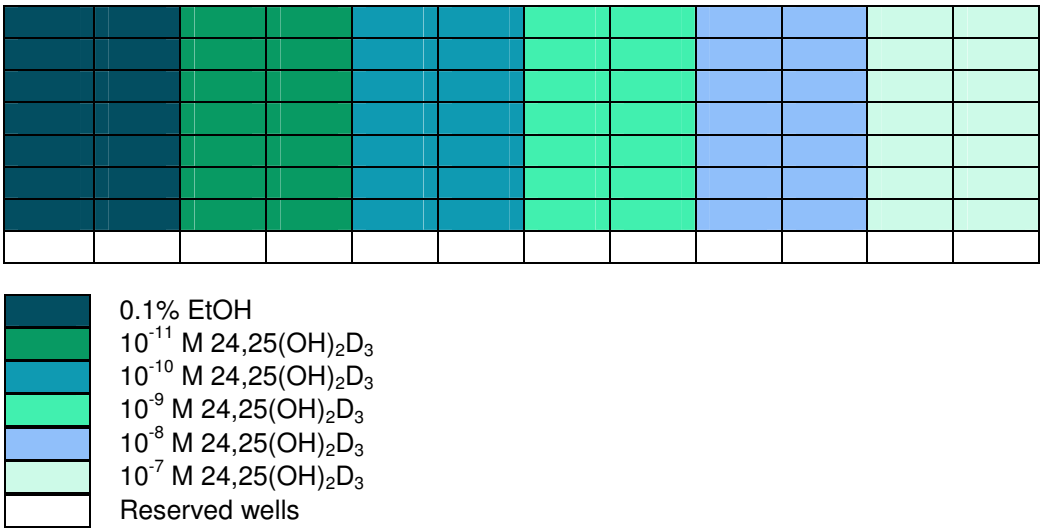
## 3.1 Cell culturing

Human prostate cancer cells from the cell line LNCaP clone FGC (ECACC, Salisbury, UK) were used for this experiment. They were grewn in a single layer in cell culturing medium (CCM): RPMI 1640 medium, supplemented with 2 mM Glutamine, 10 mM Hepes, 1 mM Na-Pyruvate, 10% Fetal Bovine Serum and 100 U/ml Penicillin-Streptomycin. The culture was kept in 37°C in a humidified atmosphere with 5% $CO_2$.

## 3.2 Treatment with $24,25(OH)_2D_3$

84 wells of a 96 well microplate (Costar 96) were each filled with 198 µl CCM containing 2 000 cells. The pipetting of cells into the microplate wells was done horizontally. The plate was then incubated in 37°C for 48 hours in order for the cells to form an attached layer in each well. To 70 of the 84 cell containing wells was then added CCM and $24,25(OH)_2D_3$ yielding a final volume of 200 µl in each well and final $24,25(OH)_2D_3$ concentrations of $10^{-7}$-$10^{-11}$ M, according to Table 1. Finally, as controls, CCM and 99.5% EtOH was added to the last 14 cell containing wells giving a final volume of 200 µl in each well and final pure EtOH concentration of 0.1%, again see Table 1. The pipetting of both hormone and EtOH into the wells was done vertically. The microplate was then incubated in 37°C for 30 minutes.

**Table 1:** Treatment of the cells with $24,25(OH)_2D_3$ using EtOH 0.1% as control. Final microplate well concentrations of $24,25(OH)_2D_3$ and EtOH, respectively.



### 3.3 Catalase assay, fluorescence measuring and computer analysis

#### *3.3.1 The Amplex Red Catalase Assay Kit, FluoStar Galaxy and GraphPad Prism 4*

The Amplex Red Catalase Assay Kit (A22180) is a very sensitive, fluorescence-based assay used for measuring of catalase activity (Molecular Probes, 2004, Appendix).

In the first step of the assay, catalase reacts with $H_2O_2$, producing water and oxygen ($O_2$) (Mueller *et al.*, 1997). In the second step, an agent called the Amplex Red Reagent (ARR) reacts with any unreacted $H_2O_2$ in the presence of horseradish peroxidase (HRP) and this generates the highly fluorescent oxidation product resorufin (Zhou *et al.,* 1997; Mohanty *et al.,* 1997). This means that the higher catalase activity in the test, the weaker the resorufin signal gets. Resorufin has a very strong absorption capacity and because of this either a fluorometer or a spectrophotometer can be used for the assay (Molecular Probes, 2004, Appendix).

In this study a spectrofluorometer, FluoStar Galaxy (BMG Lab Technologies, Germany) was used for the testing. The FluoStar measures fluorescence in Arbitrary Fluorescence Units, AFU.

The computer program GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego CA, USA) was used for the statistical analysis of the result figures.

#### *3.3.2 Assay, measuring and analysis*

After the incubation the microplate was emptied by placing Kleenex on top of it then turning it upside-down. Steps 2.1–2.10 of the Amplex Red Catalase Assay procedure was then carried out according to the Amplex Red Catalase Assay Kit protocol (Molecular Probes, 2004, Appendix). All additions in the catalase assay were pipetted horizontally into the microplate wells. The 37°C incubation time chosen for step 2.9 was 35 minutes, and the excitation and emission detection levels chosen for the fluorescence measuring in step 2.10 were set to 530 nm and 590 nm, respectively.

The fluorescence was measured at 7 time points, each separated by 15 minutes. The first measuring was done right after placing of the microplate in the FluoStar and at the same time point the built-in incubation was switched on.

The result values from the FluoStar were statistically analyzed in GraphPad Prism where graphs were drawn on basis of the result figures. The figures used for the graphs were mean values, that is, an average was calculated for the 14 replicates of each different $24,25(OH)_2D_3$ concentration as well as the 14 EtOH controls. The analyses made were a Two-way ANOVA follwed by Tukey's post-hoc test and a Linear regression analysis. The significance threshold was set to $p<0.05$.

# 4 Results and discussion

## 4.1 Dose dependent effects of 24,25(OH)$_2$D$_3$ on catalase activity

The analyses of the result values in GraphPad Prism showed that regardless of dose, 24,25(OH)$_2$D$_3$ had no statistically significant effect on catalase activity in LNCaP cells, see Figure 1. The time curves do rise some where they correspond to the $10^{-9}$ hormone concentration and this can be due to anything, however it can not be ascribed to any catalase inhibiting effect because if there had been any such effect of the hormone here, the curves would have gradually increased upwards as time passed (instead of all rising equally) along with more rapid increase in fluorescence due to higher concentration of $H_2O_2$ in those wells.

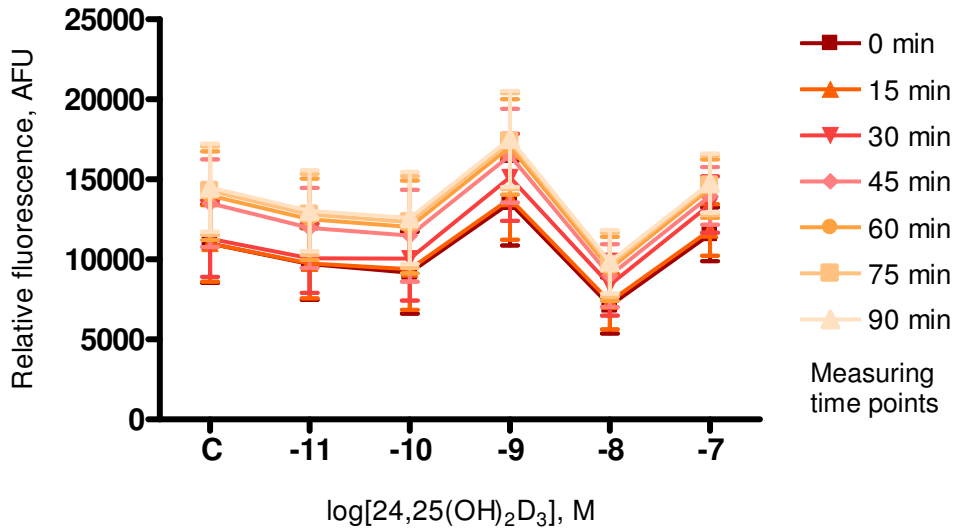



**Figure 1:** Dose- and time responses after treatment with 24,25(OH)$_2$D$_3$ were compared with help of a Two-way ANOVA followed by Tukey's post-hoc test. A fluorescence-based method was used where catalase activity was measured with Amplex Red Reagent.

To be noted is also that since all additions to the microplate except hormone and EtOH were done horizontally, this vertical digression can not have been due to cell catalase, $H_2O_2$ , HRP or ARR deviations either.

## 4.2 Time dependent effects of 24,25(OH)$_2$D$_3$ on catalase activity

The lack of catalase inhibiting effect of 24,25(OH)$_2$D$_3$ on LNCaP cells can be seen even clearer in the Linear regression analysis showing the time response, Figure 2. If 24,25(OH)$_2$D$_3$ had had any effect it had been possible to discern statistically significant differences between the coefficients of the slopes but these curves are all almost parallell, none of the digressions crosses the threshold of statistical significance. All dose curves do show slight differences in rise, however this is most likely not dependent on the varying hormone concentrations but instead due to natural breakdown of $H_2O_2$ or possibly the ARR.

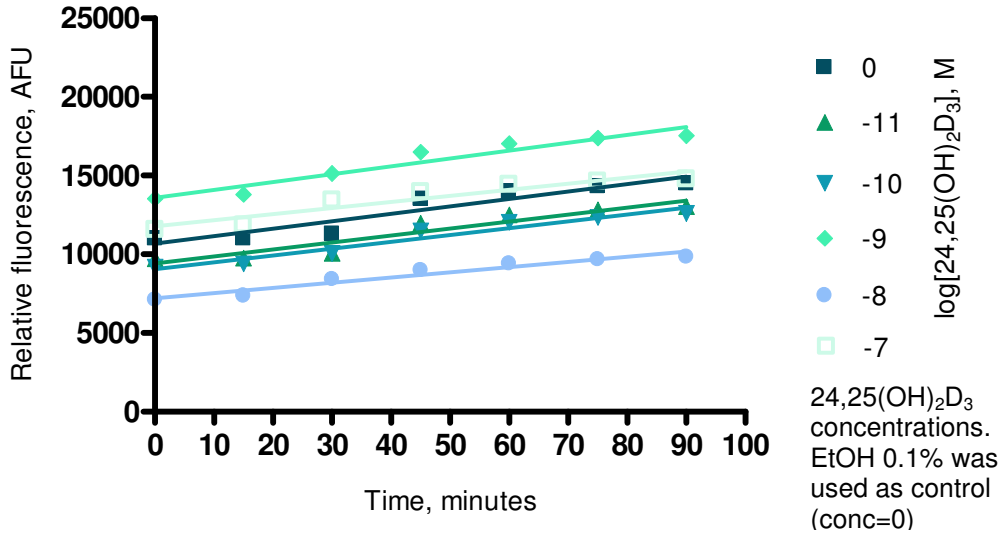



**Figure 2:** Time- and dose responses after treatment with $24,25(OH)_2D_3$ were compared with help of a Linear regression analysis.

The finding in this study that $24,25(OH)_2D_3$ does not inhibit the $H_2O_2$ reducing property of catalase contradicts a study from 2006 in which Nemere *et al.* found that $24,25(OH)_2D_3$ does inhibit catalase activity (Nemere *et al.*, 2006). This may be due to the fact that in Nemere's study chicken intestine cells were used for the testing and not LNCaP cells as in this study. Also, in contrast to the cells in this study, the cells tested on in Nemere's study were not cancerous. In other words it may be that for unknown reasons, $24,25(OH)_2D_3$ affects catalase activity in certain organs but not in others, or it may be that it affects non-cancerous tissue only. Alternatively, it could be that the hormone affects catalase in cells of certain species but not of others.

Also there is the fact that vitamin D affects our biology in many different ways. It affects many different types of tissues and there are very diverging mechanisms behind its different effects (Norman, 2004). Because of this it is still possible that $24,25(OH)_2D_3$ excerts its antiproliferative influence with help of catalase, even if not by effects on enzymatic activity. Possibly catalase may function as a receptor for the hormone but affecting a different signalling pathway, today unknown. It may also be that that other proteins function as receptors for $24,25(OH)_2D_3$.

### 4.3 Conclusion

The conclusion from this study is that $24,25(OH)_2D_3$ does not affect the biology of prostate cancer cells via inhibition of catalase activity.

Further investigations of if and in what way the hormone's binding to LNCaP cell catalase affects the enzyme or its activity are needed. Perhaps studies similar to this but experimenting with longer time periods would yield different results.

# 5 Acknowledgements

I would like to thank my supervisor, Dennis Larsson, for introducing me into the field of scientific research and for his great kindness, patience and trust when guiding me in my work.
I would also like to thank Marie Sahlberg, Jennifer Kjellerås and Samba Macauley for invaluable practical help and good suggestions.
Moreover I would like to thank my sons, Tim and Johnny, for all their love.
Finally, I would like to thank M, S and IC, for the Enlightenment.

# 6 References

Akeno, N., Saikatsu, S., Kawane, T. & Horiuchi, N. (1997) Mouse Vitamin D-24-Hydroxylase: Molecular Cloning, Tissue Distribution, and Transcriptional Regulation by 1,25-Dihydroxyvitamin $D_3$. *Endocrinology 138: 2233-2240*

Björsson, M. (2006) Does 24,25$(OH)_2D_3$ Mediate Growth Inhibition in LNCaP Prostate Cancer Cells via G Protein Coupled Signalling Pathways? Masters Thesis, School of Life Sciences, University of Skövde.

Freedman, L. P. (1999) Transcriptional Targets of the Vitamin $D_3$ Receptor-Mediated Cell Cycle Arrest and Differentiation. *J Nutrition 129: 581S–586S*

Hagberg, M. (2006) It Takes Two to Tango: Does Treatment with 24,25-Dihydroxyvitamin $D_3$ in Combination with 1,25-Dihydroxyvitamin $D_3$ or Dihydrotestosterone, Produce a Stronger Inhibition of Cell Growth in LNCaP Prostate Cancer Cells? Masters Thesis, School of Life Sciences, University of Skövde.

Haussler, M. R., Whitfield, G. K., Haussler, C. A., Hsieh, J. C., Thompson, P. D., Selznick, S. H., Dominguez, C. E. & Jurutka, P. W. (1998) The Nuclear Vitamin D Receptor: Biological and Molecular Regulatory Properties Revealed. *J Bone Mineral Res 13: 325–349*

Holick, M. F. (2005) The Vitamin D Epidemic and its Health Consequences. *J Nutr 135: 2739-2748*

Holick, M. F., Smith, E. & Pincus, S. (1987) Skin as the Site of Vitamin D Synthesis and Target Tissue for 1,25-Dihydroxyvitamin $D_3$. Use of Calcitriol (1,25-Dihydroxyvitamin $D_3$) for Treatment of Psoriasis. *Arch Dermatol 123: 1677-1683a*

Horoszewicz, J. S. (1981) *Models for Prostate Cancer* (115–132). (Murphy G. P., ed). New York: Alan R. Liss, Inc.

Krill, D., DeFlavia, P., Dhir, R., Luo, J., Becich, M. J., Lehman, E. & Getzenberg, R. H. (2001) Expression Patterns of Vitamin D Receptor in Human Prostate. *J Cell Biochem 82: 566–572*

Larsson, D., Anderson, D., Smith, N. M. & Nemere, I. (2006) 24,25-Dihydroxyvitamin $D_3$ Binds to Catalase. *J Cell Biochem 97: 1259–1266*

Lawson, D. E., Fraser, D. R., Kodicek, E., Morris, H. R. & Williams, D. H. (1971) Identification of 1,25-Dihydroxycholecalciferol, a New Kidney Hormone Controlling Calcium Metabolism. *Nature 230: 228–230*

Lou, Y. R., Qiao, S., Talonpoika, R., Syvälä, H. & Tuohimaa, P. (2004) The Role of Vitamin $D_3$ Metabolism in Prostate Cancer. *J Steroid Biochem Mol Biol 92: 317-325*

Masumoto, O., Ohyama, Y. & Okuda K. (1988) Purification and Characterization of Vitamin D 25-Hydroxylase from Rat Liver Mitochondria. *J Biol Chem 263: 14256–14260*

Mohanty, J. G., Jaffe, J. S., Schulman E. S. & Raible, D. G. (1997) A Highly Sensitive Fluoroscent Micro-Assay of $H_2O_2$ Release from Activated Human Leukocytes Using a Dihydroxyphenoxazine Derivative. *J Immunol Methods 202: 133-141*

Mueller, S., Riedel, D. H. & Stremmel, W. (1997) Determination of Catalase Activity at Physiological $H_2O_2$ Concentrations. *Anal Biochem 245: 55–60*

Nemere, I., Wilson, C., Jensen, W., Steinbeck, M., Rohe, B. & Farach-Carson, M. C. (2006) Mechanism of 24,25-Dihydroxyvitamin $D_3$-Mediated Inhibition of Rapid, 1,25-Dihydroxyvitamin $D_3$-Induced Responses: Role of Reactive Oxygen Species *J Cell Biochem 99: 1572–1581*

Norman, A. W. (2004) *Faculty Information, Norman, Anthony W.* Department of Biochemistry, University of California, Riverside. Available on the Internet: http://www.biochemistry.ucr.edu/faculty/norman.html [collected 07-06-08]

Nystrand, A. (2005) Cancer i Siffror. EPC, Cancerfonden. *ISBN 91-89446-68-2*

Sahlberg, M. (2006) The Effect of 24,25-Dihydroxyvitamin $D_3$ on Invasiveness and Proliferation of LNCaP Prostate Cancer Cells. Masters Thesis, School of Life Sciences, University of Skövde.

Winkler, S., Murua Escobar, H., Eberle, N., Reimann-Berg, N., Nolte, I. & Bullerdiek, J. (2005) Establishment of a Cell Line Derived from a Canine Prostate Carcinoma with a Highly Rearranged Karyotype. *J Heredity 96: 782–785*

Zámocký, M. & Koller, F. (1999) Understanding the Structure and Function of Catalases: Clues from Molecular Evolution and In Vitro Mutagenesis. *Prog Biophys Molecular Biol 72: 19-66*

Zhao, X. Y. & Feldman, D. (2001) The Role of Vitamin D in Prostate Cancer. *Steroids 66: 293-300*

Zhou, M., Diwu, Z., Panchuk-Voloshina, N. & Haugland, R. P. (1997) A Stable Nonfluorescent Derivative of Resorufin for the Fluorometric Determination of Trace Hydrogen Peroxide: Applications in Detecting the Activity of Phagocyte NADPH Oxidase and Other Oxidases. *Anal Biochem 253: 162-168*

### 6.1 Appendix

Molecular Probes (2004) *Amplex® Red Catalase Assay Kit Product Information.* Paisley: Invitrogen, Ltd.

*Revised: 01–October–2004*

# *Amplex® Red Catalase Assay Kit (A22180)*

**Quick Facts**

***Storage upon receipt:***
- –20°C
- Desiccate
- Protect from light

***Abs/Em of reaction product:*** 571/585 nm

## *Introduction*

The Amplex® Red Catalase Assay Kit (A22180) provides an ultrasensitive yet simple assay for measuring catalase activity. Catalase is a heme-containing redox protein found in nearly all animal and plant cells as well as in aerobic microorganisms. In eukaryotic cells it is concentrated in the peroxisomes. Catalase is an important enzyme because $H_2O_2$ is a powerful oxidizing agent that is potentially damaging to cells. By preventing excessive $H_2O_2$ buildup, catalase allows important cellular processes which produce $H_2O_2$ as a by-product to take place safely.

In the assay, catalase first reacts with $H_2O_2$ to produce water and oxygen ($O_2$).[1] Next the Amplex Red reagent reacts with a 1:1 stoichometry with any unreacted $H_2O_2$ in the presence of horseradish peroxidase (HRP) to produce the highly fluorescent oxidation product, resorufin.[2,3] Therefore as catalase activity increases, the signal from resorufin decreases. The results are typically plotted by subtracting the observed fluorescence from that of a no-catalase control (Figure 1). Using the kit, one can detect catalase in a purified system at levels as low as 50 mU/mL.

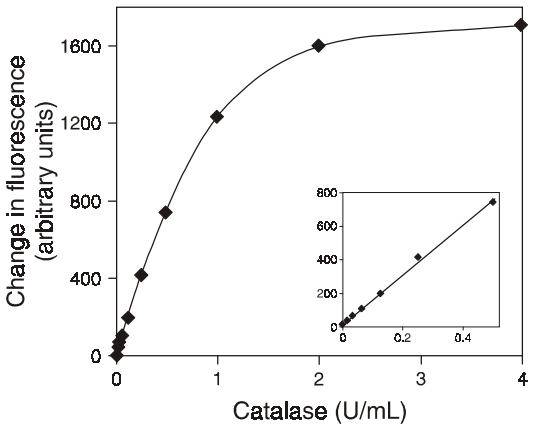



***Figure 1.*** *Detection of catalase using the Amplex Red reagent–based assay. Initially each reaction contained the indicated amounts of catalase and 20 µM $H_2O_2$ in 1X Reaction Buffer and was incubated for 30 minutes. The final reaction containing 50 µM Amplex Red reagent and 0.2 U/mL HRP and was incubated at 37°C. After 30 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. Change in fluorescence is reported as the observed fluorescence intensity subtracted from that of a no-catalase control.*

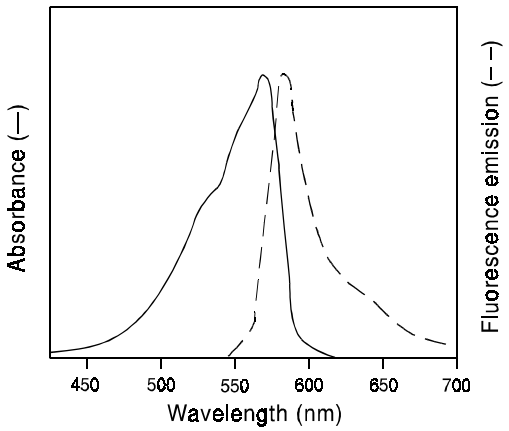



***Figure 2.*** *Normalized absorption and fluorescence emission spectra.*

Resorufin has absorption and fluorescence emission maxima of approximately 571 nm and 585 nm, respectively (Figure 2). Because the absorbance is strong, the assay can be performed either fluorometrically or spectrophotometrically.

## *Materials*

### *Kit Contents*

- **Amplex Red reagent** (MW = 257, Component A) two vials, each containing 0.26 mg
- **Dimethylsulfoxide (DMSO), anhydrous** (Component B), 500 µL
- **Horseradish peroxidase** (Component C), 20 U, where 1 unit is defined as the amount of enzyme that will form 1.0 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 20°C
- **Hydrogen peroxide ($H_2O_2$)** (MW = 34, Component D), 500 µL of a stabilized ~3% solution; the actual concentration is indicated on the component label
- **5X Reaction Buffer** (Component E), 20 mL of 0.5 M Tris-HCl, pH 7.5)
- **Catalase** (Component F), 100 U, where 1 unit is defined as the amount of enzyme that will decompose 1.0 µmole of $H_2O_2$ per minute at pH 7.0 at 25°C

Each kit provides sufficient reagents for approximately 400 assays using either a fluorescence or absorbance microplate reader and reaction volumes of 100 µL per assay.

## Storage and Handling

Upon receipt, the kit should be stored frozen at -20°C, protected from light. Stored properly, the kit components should remain stable for at least six months. Allow reagents to warm to room temperature before opening vials. The Amplex Red reagent is somewhat air sensitive. Once a vial of Amplex Red reagent is opened, the reagent should be used promptly. PROTECT THE AMPLEX RED REAGENT FROM LIGHT.

## Experimental Protocol

The following procedure is designed for use with a fluorescence or absorbance multiwell plate scanner. For use with a standard fluorometer or spectrophotometer, volumes must be increased accordingly. Please note that the product of the Amplex Red reaction is unstable in the presence of thiols such as dithiothreitol (DTT) and 2-mercaptoethanol. For this reason, the final DTT or 2-mercaptoethanol concentration in the reaction should be no higher than 10 µM.

The absorption and fluorescence of resorufin are pH-dependent. Below the $pK_a$ (~6.0), the absorption maximum shifts to ~480 nm and the fluorescence quantum yield is markedly lower. In addition, the Amplex Red reagent is unstable at high pH (>8.5). For these reasons, the reactions should be performed at pH 7-8. We recommend using the included Reaction Buffer (pH 7.5) for optimal performance of the Amplex Red reagent.

### Stock Solution Preparation

**1.1** Prepare a 10 mM stock solution of Amplex Red reagent: Allow one vial of Amplex red reagent (Component A) and DMSO (Component B) to warm to room temperature. Just prior to use, dissolve the contents of the vial of Amplex Red reagent (0.26 mg) in 100 µL DMSO. Each vial of Amplex Red reagent is sufficient for approximately 200 assays, with a final reaction volume of 100 µL per assay. This stock solution should be stored frozen at -20°C, protected from light.

**1.2** Prepare a 1X working solution of Reaction Buffer by adding 4 mL of 5X Reaction Buffer stock solution (Component E) to 16 mL of deionized water ($dH_2O$). This 20 mL volume of 1X Reaction Buffer is sufficient for approximately 100 assays of 100 µL each, with a 10 mL excess for making stock solutions and dilutions.

**1.3** Prepare a 100 U/mL solution of horseradish peroxidase (HRP) by dissolving the contents of the vial of HRP (Component C) in 200 µL of 1X Reaction Buffer. After use, the remaining solution should be divided into small aliquots and stored frozen at -20°C.

**1.4** Prepare a 20 mM $H_2O_2$ working solution by diluting the ~3% $H_2O_2$ stock solution (Component D) into the appropriate volume of $dH_2O$. The actual $H_2O_2$ concentration is indicated on the component label. For instance, a 20 mM $H_2O_2$ working solution can be prepared from a 3.0% $H_2O_2$ solution by diluting 23 µL of 3.0% $H_2O_2$ into 977 µL of $dH_2O$. Please note that although the ~3% $H_2O_2$ stock solution has been stabilized to slow degradation, the 20 mM $H_2O_2$ working solution will be less stable and should be used promptly.

**1.5** Prepare a 1000 U/mL solution of catalase by dissolving the contents of the vial of catalase (Component F) in 100 µL of $dH_2O$. After use, the remaining solution should be divided into small aliquots and stored frozen at -20°C.

### Catalase Assay

The following protocol provides a guideline for using the Amplex Red Catalase Assay Kit to measure catalase activity. The volumes recommended here are sufficient for ~100 assays, each containing a volume of 100 µL.

**2.1** Prepare a catalase standard curve: Dilute an appropriate amount of the 1000 U/mL catalase solution (prepared in step 1.5) into 1X Reaction Buffer to produce catalase concentrations of 0 to 4.0 U/mL. Use 1X Reaction Buffer without catalase as a negative control (Table 1). A volume of 25 µL will be used for each reaction. Please note that the catalase concentrations will be fourfold lower in the final reaction volume.

**2.2** Dilute the catalase-containing samples in 1X Reaction Buffer. A volume of 25 µL will be used for each reaction. Please note that the samples' catalase concentrations will be fourfold lower in the final reaction volume.

**2.3** Pipet 25 µL of the diluted experimental samples, standard curve samples and controls into separate wells of a 96-well microplate.

**2.4** Prepare a 40 µM $H_2O_2$ solution by adding 10 µL of the 20 mM $H_2O_2$ solution (prepared in step 1.4) to 4.99 mL 1X Reaction Buffer

**2.5** Pipet 25 µL of the 40 µM $H_2O_2$ solution to each microplate well containing the samples and controls.

**2.6** Incubate the reaction for 30 minutes at room temperature.

**2.7** Prepare a working solution of 100 µM Amplex Red reagent containing 0.4 U/mL HRP by adding 50 µL of the Amplex Red reagent stock solution (prepared in step 1.1) and 20 µL of the HRP stock solution (prepared in step 1.3) to 4.93 mL 1X Reaction Buffer. This 5 mL volume is sufficient for ~100 assays. Note that the final concentrations for the Amplex Red reagent and the HRP will be twofold lower in the final reaction volume.

***Table 1.*** *Sample protocol for catalase standard curve.*

| Volume of catalase solution * | Volume of 1X Reaction Buffer | Final catalase concentration † |
|---|---|---|
| 0 µL | 25 µL | 0 mU/mL |
| 6.25 µL of 1 U/mL | 18.75 µL | 62.5 mU/mL |
| 12.5 µL of 1 U/mL | 12.5 µL | 125 mU/mL |
| 2.5 µL of 10 U/mL | 22.5 µL | 250 mU/mL |
| 5 µL of 10 U/mL | 20 µL | 500 mU/mL |
| 10 µL of 10 U/mL | 15 µL | 1000 mU/mL |

* Dilutions of the 1000 U/mL catalase solution should be made in the 1X Reaction Buffer. † The catalase solution is diluted fourfold in the final reaction volume.

**2.8** Begin the second phase of the reaction by adding 50 µL of the Amplex Red/HRP working solution to each microplate well containing the samples and controls.

**2.9** Incubate the reaction for 30 minutes or longer at 37°C, protected from light. Because the Amplex Red reaction is continuous (not terminated), fluorescence or absorbance may be measured at multiple time points to follow the kinetics of the reactions.

**2.10** Measure the fluorescence or absorbance in a microplate reader using excitation in the range of 530–560 nm and emission detection at ~590 nm or absorbance at ~560 nm (see Figure 2).

**2.11** Report the change in fluorescence or absorbance by subtracting the sample value from that of the no-catalase control (see Figure 1).

## References

**1.** Anal Biochem 245, 55 (1997); **2.** Anal Biochem 253, 162 (1997); **3.** J Immunol Methods 202, 133 (1997).

## Product List

*Current prices may be obtained from our Web site or from our Customer Service Department.*

| Cat # | Product Name | Unit Size |
|---|---|---|
| A22180 | Amplex® Red Catalase Assay Kit *400 assays* | 1 kit |
| A12222 | Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) | 5 mg |
| A22177 | Amplex® Red reagent *packaged for high-throughput screening* | 10 x 10 mg |
| A36006 | Amplex® UltraRed reagent | 5 x 1 mg |

## Contact Information

Further information on Molecular Probes products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Paisley, United Kingdom. All others should contact our Technical Assistance Department in Eugene, Oregon.

Please visit our Web site — **www.probes.com** — for the most up-to-date information

**Molecular Probes, Inc.**
29851 Willow Creek Road, Eugene, OR 97402
Phone: (541) 465-8300 ● Fax: (541) 335-0504

**Customer Service**: 6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 335-0338 ● Fax: (541) 335-0305 ● order@probes.com

**Toll-Free Ordering for USA and Canada:**
Order Phone: (800) 438-2209 ● Order Fax: (800) 438-0228

**Technical Assistance**: 8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 335-0353 ● Toll-Free: (800) 438-2209
Fax: (541) 335-0238 ● tech@probes.com

**Invitrogen European Headquarters**
Invitrogen, Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Phone: +44(0) 141 814 6100 ● Fax: +44(0) 141 814 6260
Email: euroinfo@invitrogen.com
Technical Services: eurotech@invitrogen.com