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Establishment of vitamin D receptor chromatin immunoprecipitation in $CD8^+$ T cells to identify new vitamin D receptor binding sites.

By

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Abstract

Multiple sclerosis (MS) is an autoimmune neurodegenerative disorder of the central nervous system (CNS). Although, the cause of MS is still unknown, some genetical and environmental risk factor are known to be involved in the progression of MS. One of the environmental risk factors is low levels of vitamin D in serum. Vitamin D has many biological effects, and its action is mediated through its receptor vitamin D receptor (VDR, a nuclear receptor). This complex associates with retinoid X receptor (RXR), by which together will recognize specific DNA sequences, i.e. vitamin D response elements (VDREs), causing the regulation of genes that encode proteins involved in cell proliferation and differentiation of immune cells. $CD8^+$ T cells, is likely to play a role in MS pathogenesis. More than 200 single nucleotide polymorphisms (SNPs) are identified to be associated with MS. We wanted therefore, to analyse if these MS risk variants can influence VDR binding site. But before we do so, the technique used to study binding of VDR to DNA (VDR chromatin immunoprecipitation) as well as activation of $CD8^+$ T cell procedures must be optimized.

$CD8^+$ T cells were isolated from blood from healthy donors and were activated using 5 µg/ml anti-CD3 coated plate and 2 µg/ml soluble anti-CD28 antibodies for 40 hours to induce the expression of VDR. After 40 hours, VDR was highly expressed, and the cells were treated with either active form of vitamin D (calcitriol) or EtOH (vehicle control) for 3 hours. To verify the cells responsiveness to vitamin D, the expression of vitamin D responsiveness genes, *TAGAP* and *CYP24A1* were measured. Furthermore, the ChIP procedure that includes (before immunoprecipitation) protein-DNA crosslinking (incubation temperature and time), cell lysis (one step or two step) and sonication to fragment chromatin (fragmentating for 10 or 20 min) was optimized prior to be performed in two healthy donors using anti-VDR to pull down DNA regions that are bound by VDR. Anti-IgG (isotype control) immunoprecipitation was performed as negative control. PCR of regions in the *VDR*, *MYC* and *TAGAP* was used to analyse the DNA fragments purified after ChIP.

DNA regions that were pulled down by IgG were the same as VDR. So, given that our findings of ChIP are based on a limited number of experiments, the findings should be confirmed yet again doing more experiments. Therefore, immunoprecipitation part of the ChIP procedure needs to be optimized by including more materials and controls.

Sammendrag

Multiple sklerose (MS) er en autoimmun nevrodegenerativ sykdom i sentralnervesystemet (SNS). Selv om årsaken til MS fortsatt er ukjent, er det kjent at noen genetiske og miljømessige risikofaktorer påvirker MS-utviklingen. En av kjente miljørisikofaktorene er redusert vitamin D nivåer i serum. Vitamin D har mange biologiske effekter, og den virker gjennom sin reseptor, vitamin D-reseptor (VDR, som er en kjernereseptor). Dette komplekset binder retinoid X-reseptor (RXR), VDR/RXR gjenkjenner spesifikke DNA sekvenser, såkalt vitamin D reseponselementer (VDREer). Dette resulterer i regulering av gener som koder for proteiner involvert i celleproliferasjon og differensiering av immunceller. $CD8^+$ T celler spiller en rolle i MS-patogenesen. Mer enn 200 enkeltnukleotidpolymorfismer (såkalte SNPs=single nucleotide polymorphisms) er identifisert å være assosiert med økt risiko for MS. Vi ønsket derfor å analysere om disse MS-risikovariantene kan påvirke VDR binding. Men før vi gjør dette, må teknikken som brukes til å studere bindingen av VDR til DNA (VDR-kromatin-immunutfelling) og aktiveringen av $CD8^+$ T-celle prosedyrene, optimaliseres.

$CD8^+$ T celler ble isolert fra friske donorer og ble aktivert ved bruk av 5 µg/ml anti-CD3- belagt plater, og 2 µg/ml løselig anti-CD28 antistoffer i 40 timer for å indusere ekspresjonen av VDR. I tillegg ble celler aktivert med enten antistoffbelagt plate eller 1:4 (Dynabead til cell) forhold for å finne egnet aktiveringsmetode for $CD8^+$ T celle. Når VDR ble høyt uttrykt, ble cellene behandlet med enten aktiv form av vitamin D (kalsitriol) eller EtOH (negativt-kontroll) i 3 timer. For å bekrefte cellens respons på vitamin D ble uttrykket av vitamin D responsesgener, TAGAP og CYP24A1 målt. Videre ble ChIP-prosedyre som besto av kryssbinding (inkubasjonstemperatur og tid), lyseringsbuffer (ett trinn eller to trinn) og sonikering (fragmentering i 10 eller 20 minutter) ble optimalisert og utført i to friske donorer ved bruk av anti-VDR og anti-IgG (isotype kontroll) ved å trekke ned DNA-regioner som er bundet av VDR; MYC og TAGAP.

DNA-regioner som ble trukket ned av IgG, var de samme som VDR. Gitt at funnene våre er basert på et begrenset antall eksperimenter, bør funnene bekreftes med flere eksperimenter. Derfor må selve IP-delen i ChIP-protokoll også optimaliseres ved å inkludere mer materialer og kontroller.

Abbreviations

Ab	Antibody
APC	Antigen presenting cell
bp	Base pairs
CD	Cluster of differentiation
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CNS	Central nervous system
CYP	Cytochrome P
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
g	Gram
GWAS	Genome wide association study
HRP	Horseradish peroxidase
Ig	Immunoglobulin
kb	Kilo base
LAF bench	Low air flow bench
m	Milli
M	Molar
MHC	Major histocompability complex
ml	Milliliter
mRNA	Messenger RNA
MS	Multiple sclerosis
n	Nano
ng	Nano gram
nm	Nano meter
PBMS	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGK1	Phosphoglycerate kinase 1
PVDF	Polyvinylidene fluoride
qPCR	Quantitative PCR
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RRMS	Relapsing Remitting MS
RT	Room temperature
RT-	Reverse transcriptase negative control
RXR	Retinoid X receptor
SD	Standard deviation
SN	Supernatant
SNP	Single nucleotide polymorphism

TAE buffer	Tris-acetate EDTA buffer
TAGAP	T cell activation Rho GTPase protein
TBS	Tris buffered saline
TBS/T	Tris buffered saline with Tween 20
TCR	T cell receptor
Th17	T helper 12
Treg	T regulatory
VDR	Vitamin D receptor
VDRE	Vitamin D responsive elements
i.e	That is
α	Anti
µ	Micro

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1 Introduction

$CD8^+$ T cells are thought to be major effectors in the progression of multiple sclerosis (MS) (1). Activation and vitamin D receptor -chromatin immunoprecipitation (VDR-ChIP) protocols for $CD8^+$ T cells will be optimized in this thesis. The ultimate aim for the study is to identify new binding sites of VDR mediated by the active form of vitamin D ($1.25(OH)_2D$; calcitriol) and study the impact of MS susceptibility gene variants. In this section, an overview of Multiple sclerosis will be given first following vitamin D, other environmental and genetical risk factors of MS and T lymphocytes will be described.

1.1 Multiple sclerosis

- ### 1.1.1 Background

Multiple sclerosis (MS) is an autoimmune neurodegenerative disorder of the central nervous system (CNS), affecting mainly young adults, where women are more frequently affected than men (2, 3). The disease is characterized by damage of the fatty myelin sheath surrounding the nerve cells axons (demyelination) which could be due to the autoreactive T cells that penetrate through the blood-brain barrier and mediate this axonal damage in the CNS (4). Demyelination can affect the ability of the nervous system to communicate effectively resulting in physical and cognitive disability (5, 6). Relapsing-remitting MS (RRMS) is one of the subtypes of MS, which is seen in 80-85% of MS patients. These patients experience acute attacks with increase worsening of symptoms (relapses) followed by full or partial recovery (remission) and subsequent stable phases between the exacerbations (7).

Patients with MS have been diagnosed symptomatically. However, several studies are focusing on finding biomarkers for better diagnosis. Magnetic resonance imaging (MRI), to identify damaged/firm scar tissues (lesions) in the brain, which are caused by demyelination, together with neurological examination are the only tools for diagnosing MS (8, 9). Refining MRI with spinal-cord and optic-nerve imaging is one way to improve the ability to diagnose and monitor the progression of MS (8). Due to inflammation in the brain and spinal cord, oligoclonal immunoglobulin bands can be detected in cerebrospinal fluid (CSF) of 90-95% of MS patients (10, 11). These bands/antibodies arise after been released by B cells as an inflammation response.

- 1.1.2 MS as an autoimmune disease

Autoimmune diseases occur when the body's immune system start attacking its own cells and tissues. However, what triggers the immune system to attack the CNS in MS is still unknown (12). Some speculate that CNS autoimmunity can be induced by molecular mimicry. Molecular mimicry is defined as the sequence similarities among microbial (foreign) and self-epitopes, that leads to the cross-reaction of autoreactive T cells to self-epitope. This process results in the induction of several autoimmunity (13, 14).

If immune deviation, immunosuppressive cytokines (Interleukin-10 (IL-10), TGF-β) fail to disactivate the auto-autoreactive T cells that escape negative selection in the thymus (see section 1.2), these cells might be activated and start attacking self-tissues. IL-10 is an anti-inflammatory, immunosuppressive cytokine, which tends to downregulate several inflammatory messengers in the immune system, thereby maintaining normal immune response (15). Autoreactive T cells can escape the negative selection/apoptosis and induce further T cell development by expressing rearranged T cell receptor alpha (TCRα) genes (16). T cells are briefly explained in section 1.2.

- 1.1.3 Genetic and environmental risk factors of MS

MS is a complex disease with unknown cause. However, several studies show that both environmental and genetic risk factors are associated with the disease (17). Population-based twins studies have shown that MS has approximately 20-33% family recurrence rate and approximately 10-12-fold risk increase in first degree relatives (18, 19). It has been known for more than forty years that the HLA genetic region is an MS risk locus, but later, genome-wide association studies (GWAS) have identified more than 200 single nucleotide polymorphisms (SNPs) outside the HLA region that are associated with MS (20, 21). The exact location of MS risk variants is still unclear, but studies have observed that many of these variants are located near (non-coding genes) or at genes that have roles in the central immunological functions (20). MS associated gene variations can disturb transcription by being present in transcription factor binding sites and affect the folding of the chromatin.

One of the MS risk genes that overlaps with risk genes for other autoimmune diseases is Interleukin 2- receptor alpha chain (*IL2RA)* gene that encodes the alpha chain of interleukin-2 receptor (IL2R). The involvement of this gene in the pathogenesis of MS is related to the role it has in adaptive immune function; regulation of T cells (22).

Deficiency of vitamin D in the serum, smoking and Epstein Barr virus infection (23, 24) are known environmental risk factors for MS (18).

- 1.1.4 Vitamin D and MS

MS prevalence increases with the latitude, distance from the equator and is rare in subtropical areas (25). Geographical distributions and seasonal change with low exposure to sunlight leads to low levels of vitamin D ($25(OH)D_2$) in the serum (26). This strengthen the evidence that MS is strongly correlated with sun exposure. Increased sun exposure during childhood is associated with the decreased risk of MS in adolescence (23, 27). MS patients have low circulating levels of vitamin D compared to controls (28). Insufficient levels of vitamin D has a broad spectrum of actions and can affect many parts of the body and can cause diseases like autoimmune diseases, neurological diseases and coronary diseases, respiratory syncytial and virus infection, underlining the necessity of vitamin D in modulation of immune system (29, 30). It is suggested that low serum level of vitamin D is associated with the initiation of MS (31), and many studies are currently investigating this possible relationship.

A study done by Dobson R. *at el.*, shows that maternal vitamin D level during pregnancy and month of birth have significant effect on MS risk (32). In addition, a study among U.S. military personnel reveals that increased serum levels of vitamin D correlated with reduced risk of developing MS (33). A pilot study also suggests that the relapse rate reduction is seen in MS patients that were supplemented with active form of vitamin D (calcitriol) (34). It is suggested that vitamin D supplements as relevant MS treatment can improve remyelinating the damaged myelin sheath and decrease the progression of MS (28, 35, 36). Based on these considerable physiological evidence, insufficient vitamin D is one of the main environmental risk factors that has been successfully replicated.

Although the correlation of genetic factors and vitamin D in MS is still unclear, environmental factors, such as lack of vitamin D and infectious agents exert VDR regulation through epigenetic modifications, contributing in the progression to diseases like MS (37). GWAS has identified MS risk SNP in *CYP27B1* and interferes with its original function in the activation of vitamin D (see section 1.3) (22). More about vitamin D and its metabolism is described in section 1.3.

1.2 T-lymphocytes

T lymphocytes (T cells) are cells that play major roles in cell-mediated immunity. They are generated in the bone marrow and migrate to the thymus for maturation. In the thymus, these cells begin to express the TCR and two co-receptors namely, cluster of differentiation 4 and 8

(CD4 and CD8 respectively). TCR is a transmembrane receptor that is a heterodimer, containing one TCRα-chain and one TCRβ-chain and is present in 95% of T cells (38). The hypervariable region of TCR has an antigen binding site.

T cells must undergo a strict quality control through a process of recognizing self-antigens presented by a protein complex called the major histocompatibility complex (MHC). This quality control is critical as it helps the mature T cells to distinguish between self- and invading antigens. While MHC molecules are found in all types of animals, human leukocyte antigen (HLA) complex are found in humans. The MHC molecules are classified into two types; MHC class I (MHC I) is expressed on all nucleated cells while MHC class II (MHC II) is expressed only on specific immune cells called antigen presenting cells (APCs). During the selection process in the thymus, T cells develops to $CD8^+$ T and $CD4^+$ T cell. During the positive selection process, if the TCR of one T cell recognizes MHC I complex, the cell becomes $CD8^+$ T cell and stops expressing CD4. If the TCR recognizes MHC II complex, the cell becomes $CD4^+$ T cell and stops expressing CD8 (39). If the cell does not recognize either of the complexes, it undergoes apoptosis.

$CD8^+$ T and $CD4^+$ T cells are later exposed to APCs with MHC that carry autoantigens (self-peptide) and the T cells that bind with high affinity to the self-peptide-MHC will be negatively selected and must undergo apoptosis in order to avoid the development of autoreactive T cells and thus autoimmunity. Cells with TCRs that have low affinity towards self-peptide MHC complexes, are positively selected to proceed with differentiation and maintain their function in adaptive immunity (40). When the T cells have passed these steps, they are released to the circulation and resident in peripheral lymphoid organs such as lymph nodes, thymus, tonsils and spleen to defend foreign antigens. $CD4^+$ T cells are known as T helper cells (T_H cells), which secrete cytokines to regulate other immune cells to further trigger specific immune responses. On the other hand, $CD8^+$ T cells are cytotoxic T cells (T_C cells), which can destroy infected cells by secreting cytokines that attack the membrane of the infected cells and mediate apoptosis**.**

T lymphocytes play one of the main roles in the adaptive immune response alongside B cells. Upon infection, APCs such as B lymphocytes, dendritic cells and macrophages recognize antibody opsonized particles (soluble antigens) as immune complexes. Cross binding of cell surface receptors on the APCs (Fc-receptors) and an immune complex leads to internalisation of the immune complex. Subsequently, the APC breaks down the antigen into smaller peptides

that are loaded onto MHC and presented on the cell surface of the APCs. T cells recognize this MHC-antigenic complex through the TCR complexed with its coreceptor, cluster of differentiation (CD3) and triggers its response. CD3 is a protein complex that is composed of four chains and helps in transmembrane signalling.

- 1.2.1 T cell subsets

$CD4^+$ T cells are considered as the main effector in MS immunopathology, which is based on evidences from experimental autoimmune encephalomyelitis (EAE) (18, 41). EAE is an animal model for MS, an autoimmune demyelinating disease induced by injection of CNS antigens such as myelin oligodendrocyte protein, myelin basic protein, proteolipid protein or the adoptive transfer of autoreactive $CD4^+$ T cells to the animals to investigate autoimmune responses in the CNS (39). However, new evidence, especially from studies in humans, suggests that $CD8^+$T cells play a major role in the pathogenesis of MS since these cells are the predominant cell population in human MS lesions and cerebrospinal fluid (CSF) (4, 42).

Increased levels of $CD8^+$ T cells that secret proinflammatory cytokines have been observed in peripheral blood from RRMS patients in their relapsing phase compared to healthy controls and in cerebrospinal fluid (CSF) from patients in their early stage of MS (2, 39). Other proinflammatory cytokines like IL-2RA and CCL5 secreted from $CD8^+$ T are also upregulated in CSF of MS patients (43). The activity of $CD8^+$ T cells already exist in the early progression of the disease and these observations suggest that demyelination and axonal damage observed in MS lesion could be mediated by cytotoxic $CD8^+$ T cells, since $CD8^+$ T cells specifically attack myelin (44, 45). However, if this attack is mediated by primarily immune mechanisms or secondary inflammation remains unclear (9).

In addition to the $CD8^+$ T cells pathogenic role in MS, regulatory $CD8^+$ T cells, a subgroups of $CD8^+$ T cells have been observed to serve an important regulatory and a suppressive role in demyelination diseases (41, 45-47). They might contribute in limiting the disease severity in MS patients, as they were observed to suppress EAE via TGF-β- and IFN-γ-dependent mechanisms (4, 48). As it is stated in previous studies, the suppressor functions of $CD8^+$ T regulatory cells have been defective in MS patients compared to healthy controls, however, the function of suppressor $CD8^+$ T cells in MS remains a topic of considerable debate (46, 49).

- 1.2.2 T Cell activation

Upon infection, T cells require two signals in order to be activated. Signal 1 is generated when TCR/CD3 complex and either of the two co-receptors CD4 or CD8 interacts with peptides from pathogenic antigen presented on MHC molecules, while signal 2 is generated when the T cell's co-stimulator CD28 interacts with its corresponding ligands B7.1 (CD80) and B7.2 (CD86) on the surface of APCs (50, 51). T cells require both activation signals in order to be fully activated and initiate a signalling cascade finally leading responses necessary to combat infections and cancer (52-54).

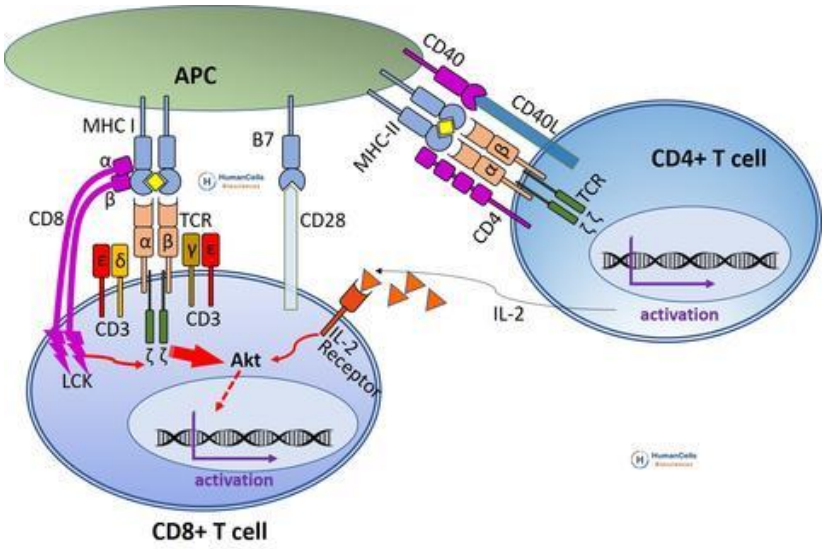


Figure 1.1: T cell activation. *The figure illustrates the activation of $CD8^+$ and $CD4^+$ T cell through the introduction of antigenic peptides from an APC. The TCR, composed of the TCR αβ and the CD3 complex, binds to the MHC-bound peptide. The figure is reproduced with the permission from HumanCells Biosciences. Available from:https://humancellsbio.com/products/human-cord-blood-cd3-pan-t-cells*

Anti-CD3/CD28 antibodies are frequently used to activate T cells *in vitro*. These antibodies interact with CD3 and CD28 on the surface of T cells, and the signalling cascade necessary for T cell activation (52, 55).

1.3 Vitamin D and vitamin D receptor (VDR)

- 1.3.1 Vitamin D metabolism

Vitamin D is a steroid hormone with two main forms, namely ergocalciferol (vitamin D_2) of plant origin and cholecalciferol (vitamin D_3) of animal origin. The source of vitamin D is from the diet, such as fish and vitamin supplements, or from UV light. The dietary sources provides a small and limited fraction of the optimal/recommended vitamin D intake (25-100µg), while the main source of vitamin D is from UV light absorbed by the skin (56, 57). In the skin, 7-dehydrocholesterol is converted to the active form vitamin D_3 (58).

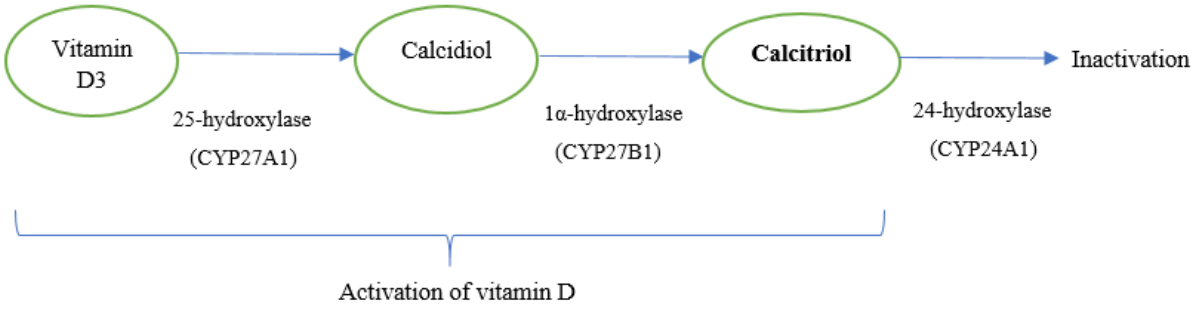


Figure 1.2: The activation and inactivation pathway of vitamin D. *The vitamin D metabolites are indicated in the circles, under the arrows, we have the enzymes that activate and inactivate vitamin D metabolites and in brackets, we have the gene names, encoding the indicated enzymes. The figure was designed by author of the thesis.*

Although, vitamin D is essential for the activation of transcriptional factors, its activation must be limited to avoid excessive expression of vitamin D mediated genes. *CYP24A1* encodes cytochrome P450 enzyme that constitute the catalysis of vitamin D molecules (i.e. Calcitriol). *CYP24A1* is expressed in most cells including bone, kidney and other cells that expresses the vitamin D receptor (VDR) and trigger VDR agonists activity in these tissues (59). The function of CYP24A1 is to limit the action of vitamin D as a negative feedback loop. Thus, increasing amount of vitamin D triggers catabolism mediated by *CYP24A1* for the prevention of cellular excessive VDR pathway response (59, 60).

- 1.3.2 Vitamin D as an immunomodulator

Vitamin D has many biological effects, as its action is mediated through its receptor, the VDR, causing regulation of several genes, including the genes that encode proteins involved in cell proliferation and differentiation, regulation of calcium homeostasis and maintaining immune defence's self-tolerance (60-62). Vitamin D with its receptor supresses the transcription of genes that encode the proinflammatory cytokines, such as IL17, IL2 and IFN-y and exerts an anti-inflammatory effect in immune cells, among others in T-lymphocytes (63).

- 1.3.3 VDR as a transcription factor

Transcription is the first stage of gene expression, which leads to the production of mRNA generated from DNA. Protein coding genes begin with a promoter sequence and ends with terminator region. RNA polymerase II is an enzyme that polymerizes DNA to mRNA. Transcription factors are proteins that binds to DNA and initiate the transcription process. These proteins have binding domains that have high affinity for specific DNA sequences, thereby directly associates with the promoter sequence or silencer (enhancer) region of the gene leading to regulation of gene expression (64).

VDR is a nuclear receptor and a transcription factor. In complex with its ligand (vitamin D), the vitamin D-VDR complex associates with retinoid X receptor (RXR; another nuclear receptor), which together will recognize specific DNA sequences, i.e. vitamin D response elements (VDRE), in the regulatory regions of genes regulated by vitamin D. The complex of vitamin D with its receptor, regulate the transcription and expression of target genes that are involved in physiological functions as described above in section 1.3.2 (65). The VDR expression and its activity plays an important role in the development and differentiation of T cells. The activity of VDR has been shown to be dysregulated by microbes in order to slow down the immune reactivity and increase their chance of survival (29, 66). Due to the function of the VDR in T cells, delayed VDR expression and its function might contribute to T cell dysregulation, subsequently causing the development of numerous diseases (67). The expression of VDR in naïve T cells is low but is increased upon T cell activation (52, 68).

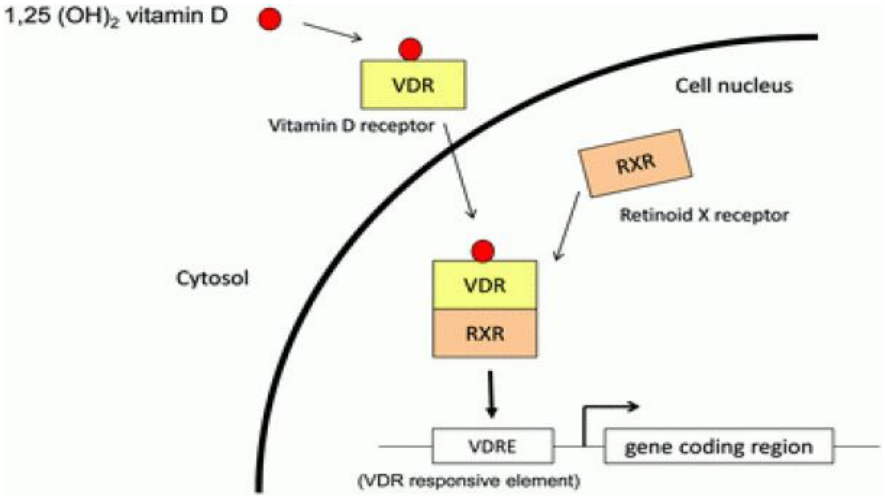


Figure 1.3: Vitamin D response is mediated through the nuclear receptor, VDR. *Vitamin D-VDR complex binds RXR and together recognize VDRE, leading to transcriptional regulation of corresponding target genes. The figure is reproduced with the permission and is available from https://selfhacked.com/blog/natural-ways-to-increase-calcitrol-and-vitamin-d-receptor-gene-expression/*

A study by Handel *et al.* show that the VDR binding in $CD4^+$ T cells are strongly correlated with the level of vitamin D in serum (69). Genes regulated by vitamin D do usually contain one or more VDREs (70).

- 1.3.4 Chromatin Immunoprecipitation (ChIP)

ChIP has been used widely for the studies of histone modifications such as methylation and acetylation. It is also used for the purpose to identify the localization of a variety transcriptional

factors binding to DNA (71). One of the early steps of the ChIP method is crosslinking (see *Figure 1.4*). The nucleophilic group of the base in DNA or amino acid from proteins forms a covalent bond with formaldehyde, a crosslinking agent (72). This crosslinking maintains the structure of chromatin. In the current thesis, VDR is the specific protein that needs to be crosslinked to the target DNA. After crosslinking and cell lysis, the chromatin of interest is precipitated out using specific antibodies against the protein of interest. After the immunoprecipitation, crosslinking is reversed and the DNA fragments that were bound to the protein of interest is purified and analysed by PCR, quantitative PCR or sequencing.

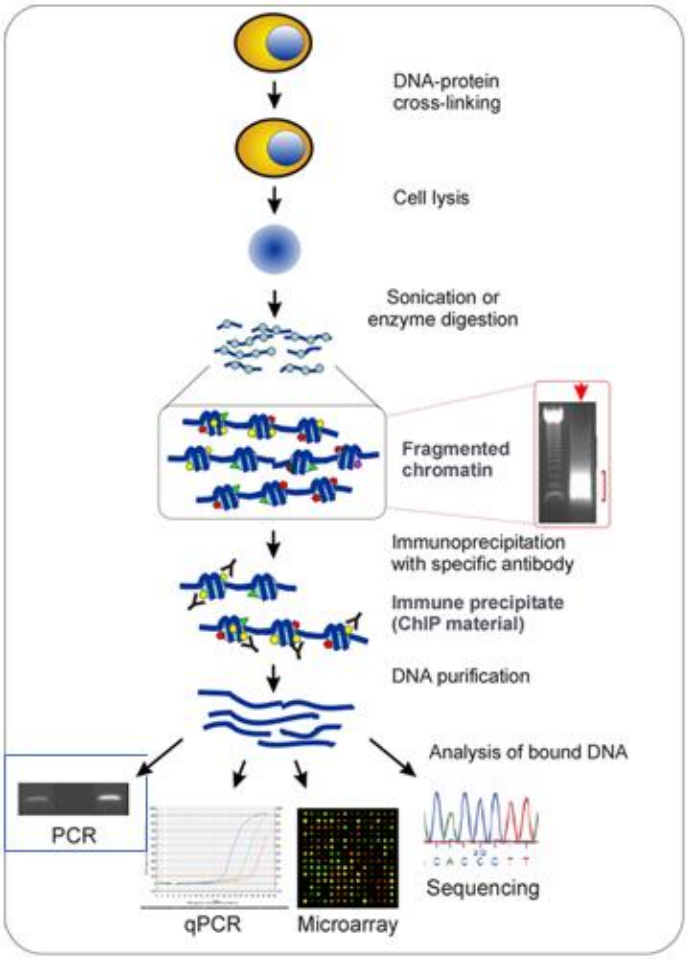


Figure 1.4: Steps of ChIP. *This figure visualises the steps to follow while performing ChIP. The figure is reproduced with the permission from creativebiomart. Available from: https://www.creativebiomart.net/blog/principle-and-protocol-of-chromatin-immunoprecipitation-chip/*

1.4 Aims of the study

The role of $CD4^+$ T cells in MS has been extensively studies, however there is a general lack of studies regarding the role of $CD8^+$ T cells. Growing evidence support the pathogenetic role for $CD8^+$ T cells in MS (1). Taking into account the current knowledge of $CD8^+$ T cells and its correlation with MS, the overall aim of this study was to perform VDR-ChIP in $CD8^+$ T cells to investigate the correlation of MS risk variants with VDR binding sites. This thesis will help to fill the gap of knowledge there is in terms of how MS genetic risk variants interact with one environmental MS risk factor, vitamin D level. The ultimate aim is to map the VDR binding sites in the genome of $CD8^+$ T cells and analyse whether MS associated genetic risk variants affect its DNA binding by the use of ChIP. In order to reach this goal, the $CD8^+$ T cell activation protocol and VDR ChIP protocol needed to be established. Therefore, the purpose of the current thesis was:

‘’Establishment of VDR-ChIP protocol for $CD8^+$ T cells to identify new VDR binding sites.’’

The sub aims were to:

- Optimize $CD8^+$ T cell activation protocol
- Analyse VDR expression in $CD8^+$ T cells
- Measure vitamin D response
- ChIP protocol optimizing including; crosslinking, cell lysis, sonication and decrosslinking protocols
- VDR ChIP combined with PCR

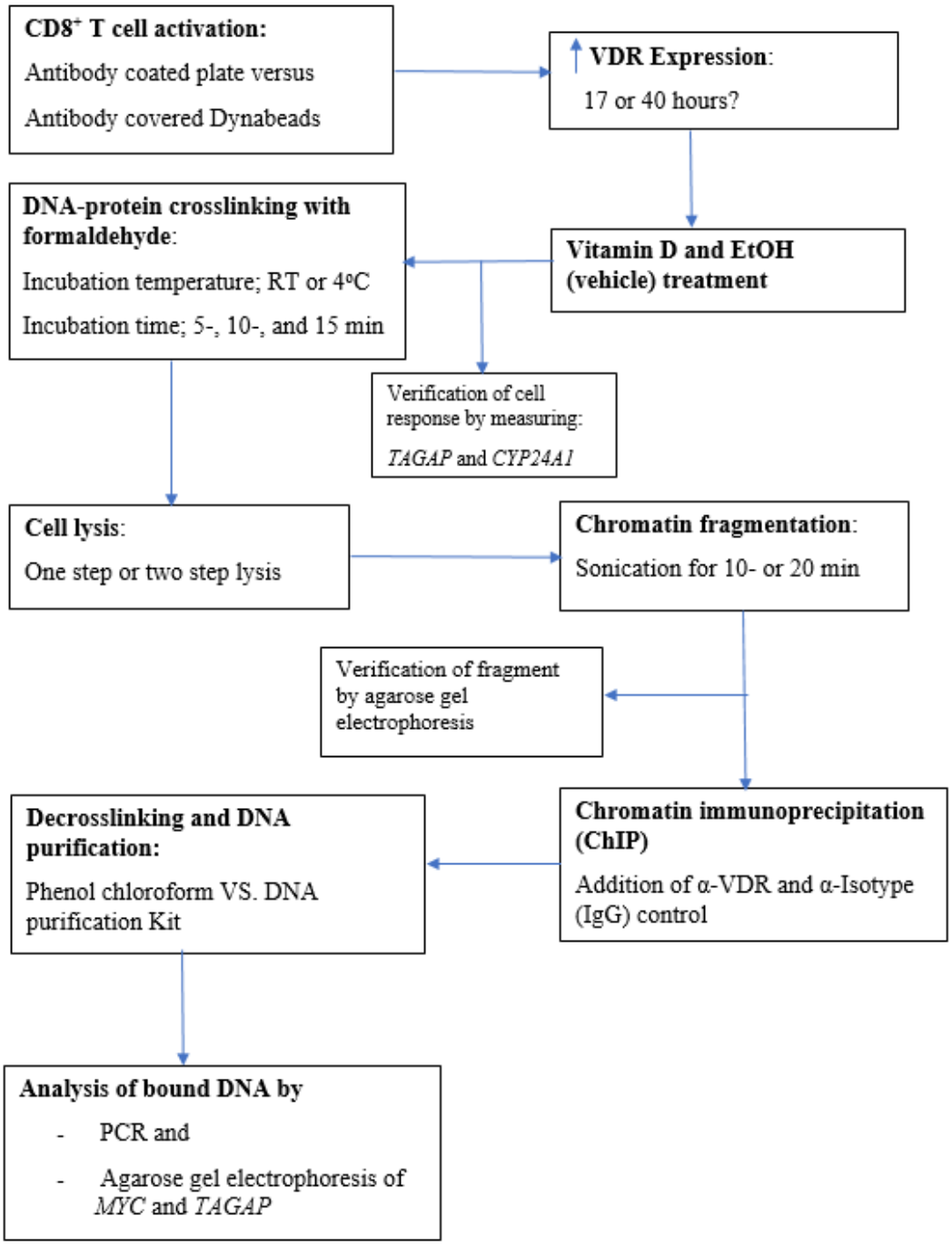


Figure 1.5: Step of ChIP procedure. *The flow chart illustrates the steps that are going to be tested in the thesis, including the steps within activation of CD8 +T cell following VDR-ChIP procedure.*

2 Materials and methods

Confidentiality and ethical considerations

Confidentiality is a key component that needs to be kept and protected in healthcare facilities including health research studies. Researchers are obligated to have responsibility and need to detail the plans they have for protecting confidentiality in a study protocol. This is done as follows:

- Informed consent is obtained from all individuals recruited to this project. These projects were approved through the approval to Hanne F. Harbo by The Norwegian Authorities and the local Ethical Committee.
- The research project has no consequences for the environment and is not putting the project participants at any risk. The study was performed in laboratories certified for the type of work described.

2.1 Isolation of peripheral blood mononuclear cells (PBMCs) and $CD8^+$ T cells

In this thesis, primary human $CD8^+$ T cells were used. Blood samples were drawn from healthy volunteers in Ethylenediaminetetraacetic acid (EDTA); anticoagulant coated tubes that preserve the blood cells morphology and avoids coagulation.

To avoid infection of the immune cells in the blood samples, all the following isolation procedures were performed in a low airflow (LAF) bench with sterile techniques. Materials that had been in contact with blood were disposed in biohazardous waste.

• 2.1.1 PBMC isolation

Procedure:

PBMC were purified from blood by density gradient centrifugation (lymphoprep; StemCELL technologies).

First the LAF-bench was disinfected with antibacterial disinfectant prior to use. 1 ml of EDTA was added in a 160 ml cell culture flask. Eight tubes containing 9 ml blood each were transferred to a medium size cell culture flask (160 ml). 10 ml of cold Rosewell Park Memorial Institute (RPMI) 1640 (Life-technologies ™) medium was used to wash off the remaining blood in the collection tubes and transferred into the cell culture flask that already contained the blood

and EDTA. The collected blood was diluted to final volume of 100 ml using RPMI1640, where it was divided in to 4 (50ml) tubes. 10ml lymphoprep (StemCell Technologies) was carefully layered underneath the blood of each tube using a syringe. The tubes were centrifuged at 800 xg for 20 mins by turning down the brake to 0 and acceleration to 1 at room temperature (RT) and the following centrifuges in this section were performed at RT.

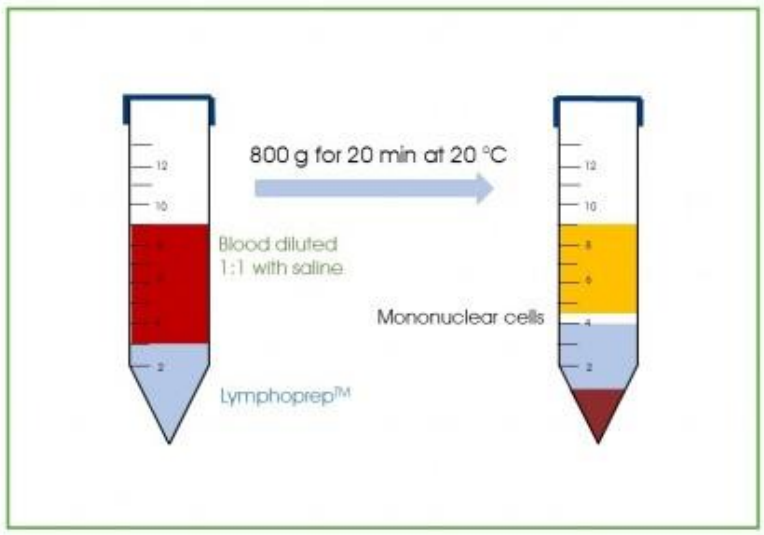


Figure 2.1: Schematic overview of PBMC isolating from whole blood using lymphoprep and density gradient centrifugation. *Erythrocytes have higher density compared to mononuclear cells, which makes the separation possible. This figure is reused with the permission from PROGEN-passion for research. Available from: https://www.progen.com/lymphopreptm-1x250ml.html*

Following density centrifugation, the mononuclear cells, which lay in the interface between serum and lymphoprep (*Figure 2.1*), were carefully transferred into two new 50 ml tubes. The PMBCs in each tube were diluted to total volume of 50 ml with PBS (Sigma Life Science) to reduce cell density. When the cells were centrifuged at 650 xg for 10 mins, the supernatant was removed, and the pellets were resuspended in 20 ml PBS. This step was repeated following centrifugation at 200 xg for 10 mins. Supernatant was removed and the pellet was resuspended completely using 20 ml PBS. 10 µl of the cell suspension was transferred to a new Eppendorf tube for cell counting see section 2.2. The 50ml tube was centrifuged at 250 xg for 10 mins. Proceed with isolation of $CD8^+$ T cells.

- 2.1.2 Isolation of CD8+ T cells

$CD8^+$ T cells were purified from PBMC (section 2.1.1) by positive selection using EasySEP Human CD8 positive selection kit (StemCell Technologies). Positive selection means that specific antibody-coupled beads were used to direct selection of the target cells; $CD8^+$ T cells, while the other cells are removed.

Procedure:

The supernatant from the centrifuged 50 ml tube (described in 2.1.1) was removed. 1ml isolation buffer (PBS w/2% Fetal Calf Serum (FCS) and 1mM EDTA) was added per 1 x 10^8 cells and transferred to a 14ml flow tube. 100µl per ml positive selection cocktail (Stem cell ™ technologies) was added to the cells following incubation for 3 mins at RT. Next, 50 µl per ml magnetic nanoparticles (RapidSphare, stem cell ™ technologies) was added to the cell suspension following incubation for 3 mins. Isolation buffer was added to a final volume of 5 ml. The tube was placed on a magnet (EasySep™ immunomagnetic column-free magnet, stem cell ™ technologies) for 3 mins. The suspension in the tube was transferred to new 14ml flow tube as $CD8^+$ T cells lay at the wall of the tube. The supernatant was used by another master student to purify $CD4^+$ T cells by negative selection. 5 ml of isolation buffer was added to wash $CD8^+$ T cells with beads and the tube was placed on the magnet for 3 mins and this was repeated one more time. After removing the isolation buffer, 4 ml isolation buffer was added and 10 µl was further transferred to a new Eppendorf tube for cell counting (section 2.2). The purity of cells was measured by flowcytometry (see section 2.4).

2.2 Cell count

Isolated PBMC and $CD8^+$ T cells were counted using TC20™ (an automated cell counter; Bio Rad) prior to further experiments. Trypan blue (BioRad) was added to the samples to distinguish live from dead cells. Dead cells do not have intact cell membrane, enabling the dye to penetrate and stain the cells, whereas living cells with intact cell membrane does not get stained.

Procedure:

10 µl of cell suspension was used to count cells. The cell suspension was mixed with 10µl trypan blue (1:1), where 10 µl was transferred to counter chamber slide (counting slides dual chamber, Bio Rad), which calculates the number of total cells and live cells/ml.

2.3 Stimulation of $CD8^+$ T cells

• 2.3.1 Titration of plate bound anti-CD3 and soluble anti-CD28 for $CD8^+$ T cell activation

Isolated cells needed to be stimulated in order to survive and be vitamin D responsive. Therefore, wells were used to stimulate cell suspension using different concentrations range from 1 – 5 µg/ml of anti-CD3 and anti-CD28 antibodies in order to find an optimal concentration for cell stimulation, (see result section 3.1).

Procedure:

Wells in a cell culturing plate were coated with 5 µg/ml anti-CD3 antibody (Invitrogen) (diluted with PBS) for 1 hr at 37ºC incubator, 5% CO_2. Meanwhile, 2 µg/ml anti-CD28 antibody (BD Bioscience) was diluted in PBS. 1 x 10^6 cells were needed per well. The cells needed for an experiment were transferred to a 15 ml tube and centrifuged at 12000 rpm for 8 min at RT. Supernatant was discarded and the cells were resuspended in preheated X-VIVO (Lonza) to a concentration of 1 x 10^6 cells/ml. After one hour at 37ºC, the PBS were removed from the wells in the plate and washed with 500 µl PBS to remove excess anti-CD3 antibodies. 100 µl of diluted anti-CD28 and 100 µl of cells (1 cells/ml) were added to each well. The plate was incubated at 37ºC for 17 hr and cells were harvested for flow cytometry and for RNA and protein analyses.

• 2.3.2 Testing different activation methods

The two activation methods used in this section are:

1) Plate bound (anti-CD3; OKT3 antibody) and soluble (anti-CD28) antibodies
2) Antibodies covered Dynabeads (anti-CD3/CD28)

The aim is to see which activation method give the best stimulation response, have low variation and leads to better survival rate. For the stimulation of cells in plate, see section 2.3.1. For the stimulation of cells using beads, the following procedure was followed:

Procedure:

Anti-CD3/CD28 coated Dynabeads (Thermo Fisher Scientific) were vortexed for 10 sec. For cell activation, 6.25 µl of Dyna-beads per 1 million cells, bead to cell ratio (1:4) were used. Dynabeads were transferred to new Eppendorf tube, washed with 1 ml of isolation buffer (PBS w/2% FCS and 1mM EDTA) and subsequently placed on a DynaMag ™-2 Magnet (Thermo

Fisher Scientific). The supernatant was removed, and this was repeated one. The beads were resuspended using 1x vol preheated X-VIVO. The cells necessary for the experiments were transferred to 15 ml tube and was centrifuged at 1200 rpm for 8 mins at RT. These cells were resuspended in preheated -VIVO (1 ml/1 million cells). Washed Dynabeads were combined with the cell suspension and transferred to wells in a plate and incubated for 17 hours (overnight) at 37ºC 5% CO_2. The activation of these cells was verified using qPCR see section 2.5.

2.4 Flow cytometry

Flow cytometry is a technology used to identify and measure cells as they are passing through a laser. It uses different scatter to identify cell size (forward scatter) and cell granularity (side scatter). Flow cytometry can also be used to identify proteins that are expressed on the cell surface by staining the cells with specific antibodies conjugated to a fluorochrome. The T cells activation marker CD69 was measured in the current thesis using this method. In addition, flow cytometry was used to measure the cell purity of $CD8^+$ T cells purified from blood.

Procedure:

First, stimulated and non-stimulated cells were prepared by adjusting the cell suspension to a concentration of $0.25x10^5$ cells/well. 25 µl of corresponding antibody solution; 1:10 dilutions of CD69-APC, IgG-APC, CD8-FITC and IgG-FITC were used to stain cells in each well and these were incubated for 30 mins on ice. Flow cytometry (Applied Biosystem by Life Technologies) was used to measure the cell surface expression of CD69. Anti-human CD69 APC-conjugated antibody (ImmunoTools) was used to stain the CD69 activation marker whereas, human IgG APC-conjugated (ImmunoTools) was used as negative isotype control. In order to analyse the purity of the isolated $CD8^+$ T, the cells were stained with FITC conjugated with anti-CD8.

2.5 RNA techniques

The cells were harvested before and after 17 and 40 hrs of stimulation and the activation response was verified using flow cytometry by quantifying the expression of the T cell active marker CD69. After verifying the activation, quantitative polymerase chain reaction (qPCR) was used to measure the level of *VDR* expression.

- 2.5.1 Extraction of total RNA

Procedure:

Cells that were harvested for RNA extraction were preserved in 350 µl RNA protect cell reagent (Qiagen) for prevention of RNA degradation and were stored at -80^{o}C. These preserved cells were thawed prior to RNA extraction using RNeasy ® Plus Mini Kit and QIA shredder columns (Qiagen) according to the manufacturer's procedure.

- 2.5.2 Measurement of RNA concentration using Nanodrop

Before proceeding with reverse transcriptase (RT) -PCR, the concentration of extracted RNA was measured using NanoDrop spectophometer - 2000c (Thermo Scientific). Nanodrop is one of the quantifiers that is used to measure the amount of extracted RNA, DNA and protein.

2 µl RNA was used to measure the concentration. RNA/DNA absorb light at 260 nm, whereas protein and organic components containing phenol rings absorb light at 280 nm and 230 nm respectively. By measuring the absorbance ratio between 260/280 and 260/230, the purity of the nuclei acids can be determined, and protein contamination and carryover of components (such as phenol) can be detected. These carryover components are known to lower the reaction efficiency and inhibit enzymatic reactions in PCR. Nuclease-free water (RNA was eluted with) was used as blank.

- 2.5.3 Reverse transcriptase PCR (RT-PCR)

Polymerase Chain reaction (PCR) is a technique that is used in molecular studies to amplify a specific fragment of DNA from a single copy to thousands and millions of copies. RT-PCR is a technology that can reversely transcribe RNA to complementary DNA (cDNA) using an enzyme known as reverse transcriptase. This is routinely done prior to real time quantitative PCR (qPCR) analysis as RNA cannot be used as template in qPCR, due to the exposure of broad temperature ranges required within the gene expression analysis.

Procedure:

All RNA samples were diluted in nuclease-free water to a common RNA concentration and subjected to cDNA synthesis using cDNA Synthesis Kit for RT-PCR (Thermo Scientific) using the following reagents:

Table 2.1: Setup of RT-PCR performance and the reagents needed to the process.

Reagents	**Volume (µl)**
5x Reaction Mix	4
Maxima Enzyme Mix	2
RNase free water	Up to 20 µl
Template	1 pg – 5 µg RNA
Total Volume (µl)	**20**

A reverse transcriptase negative control (RT- negative control) containing all components but using water instead of reverse transcriptase was included. The purpose of using RT- control was to confirm that the RNA was free from genomic DNA (gDNA). In addition, a water control (WC); non-template control which contained all components except RNA template, was also included to evaluate the reagents contamination.

The cDNA was amplified using following steps:

Initial step 10 min 25^{o}C
Reverse Transcriptase step 30 min 58^{o}C
Inactivation step 5 min 95^{o}C
The generated cDNA was stored at -20^{o}C until performing qPCR.

- 2.5.4 Quantitative Real Time PCR (qPCR)

To study the quantity of expressed genes, methods such as qPCR are used. qPCR is a based on a PCR technique that measures the production of expressed genes in real time; *VDR* and *CD69* in this thesis, by doubling at each cycle. In the current thesis sequence-specific DNA probes; TaqMan® were used for detection of amplification in real time. These are single stranded DNA probes coupled to a fluorescent dye (reporter) and a quencher. The quencher inhibits and ensures a fluorescence signal from reporter to be detected only when a DNA polymerase elongates the target sequence by hybridizing to its complementary sequence, degrades the probe and the reporter is released from quencher. Therefore, the signal that is released is proportional to the number of PCR products that is generated.

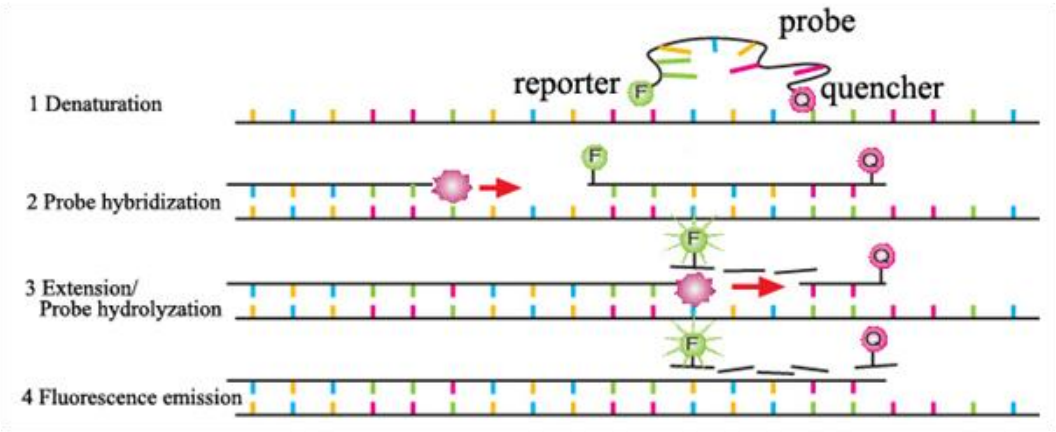


***Figure 2.2: Overview of TaqMan probe detection**, when the florescence is released during the extension. The figure was reused with permission and is available from: https://www.sinobiological.com/taqman-probe-qpcr-method-cro-service.html*

The qPCR standard curve method requires standard curve dilution series which contains known concentration of RNA and is used to predict the concentration of samples by creating a standard curve; a graph where the unknown concentrations are interpolated within. Standard solution (S1) is the first point in the standard curve (the highest concentration) and is generated during the RT-PCR step following a 1:2 in a 7-point dilution series (S1-S7) in advance of performing qPCR. The standard curve is amplified together with the samples to determine their concentration and to so do, the samples must be within the standard curve range. In addition, the standard curve can be used to control that the cDNA synthesis is not saturated, ensure that the reaction is approximately 90-110% efficient and that the PCR amplicon increase with every 3,3 cycles, resulting in a slope around -3.3.

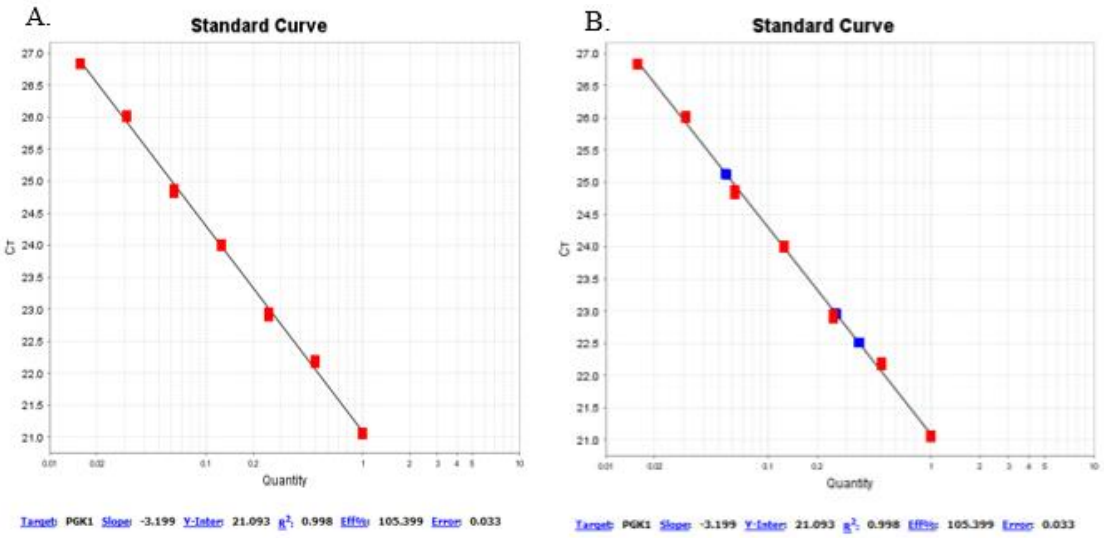


***Figure 2.3: Relative standard curve. A**. Representative standard curves from this thesis composed of a seven-point standard with 1:2 dilution series and including known concentration of the housekeeping gene PGK1 (red dots) for predicting the concentration of target gene expression (blue dots) in **B.** PGK1*

gene is labelled with FAM detection probe. This curve has 105% efficiency and the PCR amplicon increases with every 3,2 cycles leading slope to be approximately -3,2.

Procedure:

These qPCR experiments were prepared according to the instruction delivered by Applied Biosystems (Life Technologies). In one of the reactions, water was used as substitute for cDNA and served as negative control. Duplicates of 10 µl qPCR reaction were loaded in a 384 – well plate. The plate was centrifuged at 2000 rpm for 5 mins at RT and the PCR program used 40 cycles on Applied Biosystems Viia™ 7 Real Time PCR system (Life Technologies).

Normalized targets = Target / reference gene (PGK1)

2.6 Protein techniques

• 2.6.1 Protein extraction

Western blotting was used to verify the protein expression of the VDR in $CD8^+$ T cells before and after activation. The cells must be lysed to expose the protein content of the cells.

Procedure:

Cells were centrifuged at 300 xg for 5 mins at 4°C. The supernatant was removed, and the cells were washed twice with 1 ml PBS following centrfugation. Supernatant was completely removed after the last wash and 20 µl of SDS loading buffer with β-mercaptoetanol per 1 x 10^6 cells was added to the cells prior to sonication (UP100H Ultrasonic Processor, Hielscher). These samples were stored at -20°C until proceeding with SDS-PAGE.

• 2.6.2 SDS-PAGE

Dodecyl Sulfate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE) is a method utilizes SDS; an anionic detergent that denatures the tertiary structure of proteins by linearizing the primary structure and adds a negative charge to the proteins, by which the protein can migrate according to their molecular weight towards the positive pole in an electric field.

Choosing the right percentage of polyacrylamide for separation of proteins with a certain molecular weight is an important factor to take into consideration to make sure optimal separation of protein bands. High percentage of polyacrylamide increase the size of pores and leads to slower migration of the proteins, whereas using lower percentage leads to faster migration. To predict the molecular weight of migrated protein, a molecular standard that includes proteins with known sizes was also added to the gel.

Procedure:

The samples (from section 2.6.1) were preheated for 5 mins at 96^{o}C. A gel (Criterion ™ TGX™ Precast Gels, Bio-Rad) was placed in an electrode assembly and the comb of the gel were carefully removed. The assembly was filled with 1x SDS running buffer (Sigma-Aldrich). 10 µl of a molecular standard (Precision Plus Protein ™ dual colour standard (Bio-Rad)) was loaded to the first well and 20 µl of samples that contains lysate from 1 x10^{6} cells were loaded in the following wells. The gel was first run at 120 V for 15 mins, followed by 150 V for approximately 50 mins.

- 2.6.3 Western blotting

Western blotting is a method used to identify a specific protein from a protein mixture. The separated proteins (from section 2.6.2) are transferred to a Polyvinylidine fluorine (PVDF) membrane (Immune-blot ®, BioRad). In order to prevent non-specific binding of antibodies to protein in the membrane, the membrane is blocked by incubating it with 3% skimmed milk in TBS/T for 2 hours or overnight at 4°C**.**

The membrane was subsequently incubated with primary antibody that is specific to the target protein; VDR. A secondary antibody linked with the enzyme Horseradish Peroxidase (HRP) was then added to the membrane and recognizes the primary antibody. The HRP produces light when its substrate is added to the membrane. The light produced is proportional to the amount of target protein (VDR) on the membrane. Anti-actin (produced in rabbit) was used as a loading control, as it is stably expressed in all eukaryotic cells.

Procedure:

Protein transfer:

After SDS-PAGE (see section 2.6.2), the gel was washed with dH_2O and placed in an assembled transfer pack (Trans-Blot Turbo blotting system, Bio-Rad). The transfer was run at 25 V for 7 mins. The membrane was washed in dH_2O and stained for approximately 1 min using premixed Ponceau Red Staining (Sigma-Aldrich) to confirm that the proteins had transferred from the gel to membrane. This staining solution was discarded, and the membrane was washed several times using mq-H_2O until the red colour from the background was completely washed away.

Detection with antibodies:

First, the membrane was blocked using 3% skimmed milk for 2 hours at RT. Primary antibody (mouse anti-VDR or rabbit anti-actin) was diluted 1/350 and 1/3000 respectively in 3% skimmed milk and was added to the membrane and incubated at 4^{o}C overnight. Next, the membrane was washed 4x15 mins in TBS/Tween and incubated with secondary antibody (goat anti-mouse for VDR detection and goat anti-rabbit HRP for actin detection) both diluted 1/ 5000 in 3% skimmed milk, for 1 hour at RT. The membrane was washed 4x15 mins in TBS/Tween and placed in a plastic beaker. 2 ml of each ECL SuperSignal ® solutions (ThermoFisher Scientific) in 1:1 ratio was mixed prior to adding the solution to the membrane and incubated for 5 mins. The membrane was covered with plastic film by removing all bobbles and placed in the developer cassette (ChemiDoc™ Touch Imaging System, Bio-Rad) for developing for approximately 1 hour for VDR detection and approximately 2 mins for actin detection.

Stripping and reprobing of membrane:

Primary and secondary antibodies from earlier section (Detection with antibodies) were washed off from the membrane prior to reprobing the membrane with actin specific antibodies.

Procedure:

The membrane was stripped using Blot stripping buffer (Thermo Scientific) for 15 mins at RT. The remaining steps are described above under ‘’ detection of antibodies’’.

2.7 Vitamin D treatment of $CD8^{+}$ T cells

Vitamin D in the form of calcitriol (active form of vitamin D) is diluted 1:100 to a final concentration of 10 nM vitamin D in preheated X-VIVO. 1 µl of diluted vitamin D was added per 1 ml cell suspension. Since vitamin D is very toxic in high concentration, the dilution was generated in a fume hood. EtOH served as negative control, was also diluted 1:100 in preheated X-VIVO, where 1 µl was added to the cells in a separated well. The activated cells treated with either vitamin D or EtOH were incubated at 37^{o}C for 3 hours prior to harvesting. RNA was extracted from these harvested cells and the full protocol is stated in section 2.5.

2.8 Chromatin immunoprecipitation

• 2.8.1 Crosslinking

Procedure:

70 µl formaldehyde (36.5 % stock, Sigma-Aldrich) was added to the 2.5 ml of cell suspension. The samples were incubated for 5-,10- and 15 min at either 4ºC or RT on a roller. 135 µl of glycine (Sigma-Aldrich) was added to the samples following incubation for 5 min at RT to deactivate the formaldehyde. These samples were centrifuged at 500 xg for 10 min at 4ºC. When the supernatant was discarded, the pellet was resuspended in 2 ml ice-cold PBS twice.

• 2.8.2 Cell lysis

Procedure:

Cell lysis and nuclear buffers with 40 µl of 25% protease inhibitor (Pi; Roche) and 10 µl phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich) were first prepared. 1 ml of cell lysis was added to the sample and incubated for 10 min on ice. The samples were centrifuged at 300 xg for 3.5 min at 4ºC. The supernatant was removed following the addition of 100 µl nuclear lysis buffer and incubation for 10 min on ice. Additional nuclear lysis buffer was added to obtain a final volume of 300 µl. The lysate was transferred into 1.5 ml Microtube for Bioruptor Pico (Diagenode) for DNA fragmentation using sonication.

• 2.8.3 Sonication of chromatin

Procedure:

The chromatin was fragmented/sonicated for 30 sec ON/OFF for 10 min at 4ºC using the Bioruptor Pico. The samples were transferred back into 1.5 ml Eppendorf tube and was centrifuged at 10 000 xg for 10 min at 4ºC. In order to avoid the upper layer and cell debris in the bottom of the tube, approximately 220 µl of supernatant (chromatin) was transferred into a new Eppendorf tube to perform immunoprecipitation (see section 2.9), where 10 µl of this sample was saved for the analysis of the fragment size on gel.

• 2.8.4 Preparation of chromatin prior to fragment analysis

Procedure:

10 µl from section 2.8.3, was used to assess chromatin fragment size by agarose gel electrophoresis before ChIP. 1 µl RNase (500 µg/ ml stock) was added to the sample and incubated in a thermo block at 37ºC for 20 min. 190 µl of elution buffer containing 1% SDS

was added to the sample prior to the addition of 5 µl proteinase K (from 2 mg/ml stock) and incubation on a thermo mixer at 68ºC, 500 rpm for 1 hr.

Purification of the ChIP DNA:

The fragmented DNA was purified and washed for removing any other cellar components before fragment size analysis. It was performed as follows:

Procedure:

200 µl (1x vol) phenol-chloroform isoamylalcohol (pre-mix at 24:24:1 vol ratios; Sigma Life Science) was added to the fragmented chromatin from section 2.8.4. The sample were mixed thoroughly followed by centrifugation at 12900 rpm for 5 min at RT. This solution separated DNA from other cellular components. 200 µl of the upper phase (containing the DNA) was collected using a pipette and transferred into a new tube and 1 x vol of chloroform and isoamylalcohol (pre-mixed at 24:1; Sigma Life Science) was added to the sample. The samples were mixed thoroughly prior to centrifugation at 12900 rpm for 5 min at RT. 140 µl of the upper phase was carefully collected and transferred into a new tube. 14 µl of 3M Sodium Acetate pH 7.0 (BioXtra) was added to the sample and was mixed briefly. 3.1 µl of Glycogen (20 µg/ µl; Invitrogen) and 393 µl ice cold 96 % EtOH was added and the sample was incubated for 2 min. This sample was placed at - 80ºC overnight for the precipitation of DNA. Next, the sample was centrifuged at 20000 xg for 15 min at 4ºC. The supernatant was discarded, and the DNA pellet was left to dry for approximately 15 min at RT by leaving the tube open. The DNA pellet was dissolved in 20 µl MQ H_2O at RT for 1-2 hr. The DNA, by this point was ready to proceed with the fragment size analysis by agarose gel electrophoresis.

- ### 2.8.6 Analysing the size of fragmented DNA by agarose gel electrophoresis

Agarose gel electrophoresis is a standard method that is performed to separate, identify and clean DNA fragment and proteins. The method is easy to use and can separate fragmented DNA down to 50 base pairs. DNA is a negatively charged molecule and will thereby migrate to the positive pole when it is loaded on a gel in an electric field. The gel has pores that interferes with the migration, so that the bigger DNA fragments migrate slower and can easily be separated from the smaller fragments that migrate faster and can be seen in the bottom layer of the gel. The concentration of the agarose gel contributes in leading a clear separation among the DNA fragments. The higher voltage the gels are exposed to, the faster the fragments migrate.

In order to visualize the DNA in the gel, ethidium bromide (EtBr) is added. EtBr intercalates with double stranded nucleotides of DNA so that DNA can be visualized under the presence of ultraviolet light (UV-light).

Procedure:

In this thesis, Tris-Acetate-EDTA buffer (TAE) had 50x start concentration and was diluted into 1x before use. 1.5 % agarose gel was used. It was generated by mixing 0.75 g agarose powder with 50 ml of 1xTAE buffer. This reaction mix was heated in the microwave until the agarose powder is completely dissolved. This solution was cooled down to almost 55ºC prior to addition of 2.5 µl EtBr (**s**tock 10 mg/ml) for visualization. This gel mixture is poured into a gel-casting tray and then the comb is put in the gel to make wells where the samples can be loaded. The gel was left to solidify for approximately 30-45 minutes. The comb was removed and 1x TAE buffer was added to cover the gel. 3.3 µl 6x loading buffer (LB) was added to the 20 µl samples prior to loading them into the wells. Loading buffer is a buffer that contains glycerol, which help the samples to sink into the wells of the gel. First load 10 µl DNA ladder (Gene Ruler 100 bp and Gene Ruler Low Range (contains fragment length down to 25-700 bp used in section 2.10)) in the first well and then load the samples in corresponding wells. DNA ladder is a molecular weight standard with known sizes, which help to estimate the length of target fragmented DNA.

The gel is placed in an electric field using 80 V at 240 mA for 40-45 minutes. After the gel was run, it was placed in a gel doc (GeneGenius Gel Light Imaging system) for visualization of migrated DNA fragments.

- 2.8.7 Immunoprecipitation

Specific antibodies against VDR were used to pull out DNA fragments associated with VDR. Four primer pairs were used as positive controls expected to cover regions bound by VDR in a study done by Handel *et al*., and they recognize regions in the following genes: *VDR, TAGAP* and *MYC* (69) . Input chromatin was used as a control. This is a control that contains chromatin which did not undergo any selection for fragments associated with the binding of target gene, and this is used to quantify and normalize the variation of immune precipitated target genes from the starting material.

Procedure:

Chromatin dilution;

Fragmented chromatin; 200 µl from section 2.8.3 was divided into two samples to be used immunoprecipitation with α-VDR and α-IgG, respectively. This means that each sample will contain 100 µl each and each were diluted 1:9 using RIPA buffer without SDS, leading to a total volume of 1 ml each. 100 µl of these dilutions was saved as input prior to antibody addition. The two tubes with remaining 900 µl each were placed aside on ice while coupling of protein A Dynabeads (Invitrogen) to α-VDR and α-IgG.

Coupling of antibodies to magnetic Dynabeads:

The protein A Dynabeads were vortexed right before use. 40 µl of Dynabeads per ChIP (VDR and IgG) was transferred to two new Eppendorf tube. 2.5x vol of RIPA buffer with SDS was added to each tube and mixed briefly. This mixture was incubated for 1 min and the buffer was discarded as the tube was still on the magnet (DynaMag ™-2 Magnet (Thermo Fisher) and this step was done twice. The beads were resuspended in 400 µl RIPA buffer with 0.1% SDS (per ChIP). The beads were incubated with corresponding antibodies; 27.5 µl α-VDR (200 µl/ml stock; Santa Cruz Biotechnology) and 12.5 µl α-IgG (200 µl/ 0.5 ml stock; Santa Cruz Biotechnology). The Dynabead antibody mixtures were incubated for 2 hr at 40 rpm on a rotator in the cold room.

The tubes were shortly snapped to bring down what was trapped in the lid and was placed on a magnet on ice for 1 min. The supernatant was removed as the tube was still on the magnet. The corresponding bead-antibody complexes were resuspended by each of the corresponding samples which contained the 900 µl. The lysates were incubated with antibody-coated beads overnight at 40 rpm on a rotator in the cold room.

Washing (on ice):

The samples were shortly snapped and placed on magnet on ice for 2 min. The supernatant was removed while the tubes were on magnet. 2 ml RIPA buffer with SDS was added to each sample and was incubated for 4 min at 40 rpm on rotator in the cold room. This step was done three times, but before the last wash, 1 ml RIPA buffer was added to each sample, mixed briefly using pipette and transferred to a 1,5 ml tube and placed on the magnet and lastly, the supernatant was discarded.

Crosslink reversal, RNase treatment and DNA elution:

290 µl and 190 µl elution buffer containing 1% SDS was added to the ChIP samples and Input samples respectively. 10 µl RNase (Roche) was added later to each sample. The ChIP and Input samples were then incubated at 37ºC for 20 min. 2 µl proteinase K (20 mg/ml stock) was supplemented to each sample and the samples were incubated at 68ºC, 1300 rpm for 6 hours. Another 2 µl was added to the samples after 2 hrs during the incubation time.

Purification and elution of DNA using MicroChIP DiaPure columns (Diagenode) was done according to the manufacturer's instruction.

2.9 Polymerase chain reaction (PCR)

The purpose of this section is to generate many copies of the immune precipitated DNA from the eluted DNA using the 4 primers pairs covering gene region that have been shown to be bound by VDR in $CD4^+$ T cells.

Procedure:

Table 2.2: Reagents for the generation of master mix for PCR.

Reagents	**Final amount**	**Volume per reaction**	**Volume for 56 reations (master mix x56,5)**
Forward primer	20 µM (diluted 1:100)	4 µl	
Reverse primer	0,2 µM (diluted 1:100)	4 µl	
Deoxynucleotide (dNDP)	0,2 µM (diluted 1:100)	4 µl	226 µl
10xPCR buffer -Mg (Invitrogen)	10%	2 µl	113 µl
50 mM $MgCl_2$	1,5 mM	0,6 µl	33,9 µl
DNA template	8, 2 and 0,5 ng	2 µl	
Taq Platinum DNA polymerase (Invitrogen)	2U /rxn	0,08 µl	4,52 µl
Autoclaved MQ water	Add to 20 µl	3,32 µl	187,6 µl

Table 2.3: Primer sequences (5'-3') of 4 target genes.

VDR	Forward	5'-GAG AGG GAG TCG TGG GTT TC-3'
	Reverse	5'-GGC TTT GCT GGT GAC ACA TC-3'
MYC	Forward	5'-GGA GAT CCG GAG CGA ATA GG-3'
	Reverse	5'-CGG CAA GGG TTG CGG AC-3'
TAGAP1	Forward	5'-ATG CCA GCT GTC GTA GTA GG-3'
	Reverse	5'-ACT CTG GCT AGA GAC CCT CC-3'
TAGAP2	Forward	5'-CAG CTG TCG TAG TAG GTG CTT-3'
	Reverse	5'-CGT GAA GGC TGC ACC TTT TT-3'

GAPDH Intronic 2	Forward	5'-GGG ACG CTT TCT TTC CTT TC-3'
	Reverse	5'-ATC CGG ACA GGG ATG CAC-3'

Water, PCR buffer, $MgCl_2$, dNTP mix, polymerase enzyme was mixed in Eppendorf tube and 10 µl of this master mix was transferred to each PCR tube. 2 µl of template and 4 µl of each forward and reverse primers were added to corresponding tubes leading to a final reaction volume of 20 µl.

PCR was run in following program:

1 cycle: Denaturation 95°C for 4 minutes

40 cycles: Denaturation 95°C for 1 minute

Annealing 55°C for 30 seconds

Elongation 72°C for 1 minute

1 cycle: Elongation 72°C for 7 minutes

Agarose gel electrophoresis was performed for each sample to find out which gene from *Table 3.* was/were bound by VDR. The procedure is stated in section 2.8.6.

2.10 Statistical analysis

Data from experiments were expressed as mean±SD and student's paired t-test was used to analyse whether there is any statistically significant between two groups. The level of statistical significance was set at 0.05.

3 Results

In a study done by Berge. T *at el.*, freshly isolated $CD4^+$ T cells had low or no VDR expression (52). Therefore, we hypothesized that freshly isolated $CD8^+$T cells also needed to be activated in order to express VDR. The expression of VDR was assessed at mRNA and protein level in $CD8^+$T cells using TaqMan probes and immunoblot analysis respectively. Prior to these experiments, the optimal concentration (section 3.2) of antibodies that gave high cell stimulation and improved cell viability was determined. But, before that, cell purity was examined in the following section 3.1.

3.1 Cell purity

Positive selected $CD8^+$T cells were isolated from whole blood as described in 2.1.2. Cell purity was measured to verify if these isolated $CD8^+$T cells were indeed $CD8^+$T cells. Purity was measured routinely by flow cytometry and the purity result (of all isolated $CD8^+$T cells) is shown in *Table 3.1*. One representative experiment (Donor ID, 3), out of eight is viewed in *Figure 3.1* to show the gating setup prior to performing cytometry in *Figure 3.1 A and B.*

Table 3.1: The purity of $CD8^+$T cells after isolation measured by flow cytometry. *$CD8^+$T cells isolated from different donors at different time point. The cell purity seems to vary among donors.*

Donor ID	Cell purity (CD8-FITC (%))
1	82
2	84
3	80
4	64
5	56
6	84
7	75
8	75

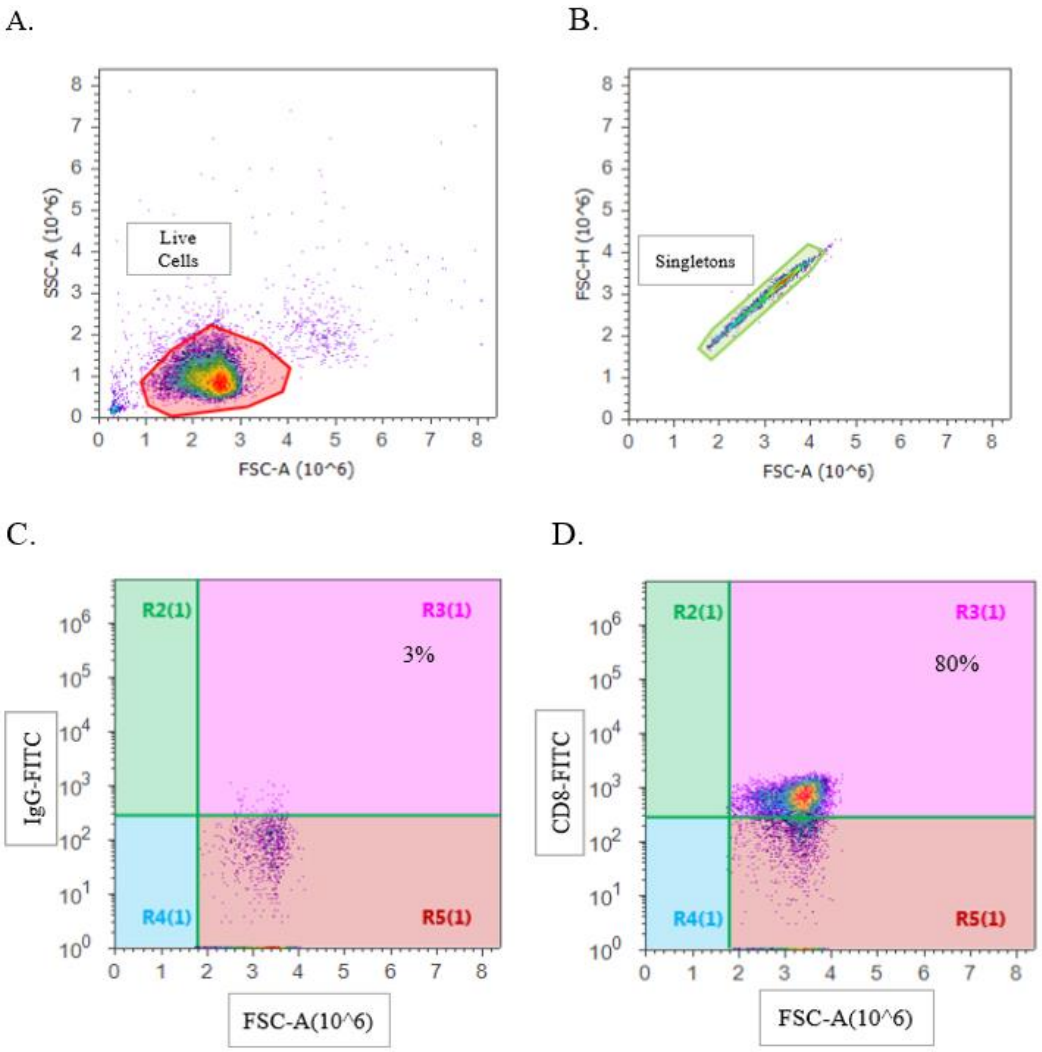


Figure 3.1: Cell purity measurements of CD8⁺T cells using flow cytometry. *After positive selection of CD8⁺T cells, cells were stained with FITC-conjugated anti-CD8 antibody or an isotype control, and cell purity was measured by flow cytometry. Forward scatter area (FSC-A) versus side scatter area (SSC-A) are used to select live cells in* ***A.*** *followed by FSC-A versus FSC-H scatter used to select singletons (single cells) from clustered cells in* ***B.*** *IgG-FITC staining is shown in* ***C.*** *and CD8-FITC staining is shown in* ***D.*** *Numbers in upper quadrant indicate the percentage of cells that are live, single and positive cells for the indicated antibody used for staining.*

3.2 Titration of anti-CD3/CD28 antibodies for CD8⁺ T cell activation

For cells to be vitamin D responsive, the VDR needs to be expressed prior to vitamin D addition. But it has previously been reported that VDR expression is low in naïve T cells, and that it is upregulated upon T-cell activation (52). Therefore, we wanted to examine whether that was the case also for CD8⁺T cells purified from blood. In order to determine the optimal antibody concentration to use in the downstream activation experiment, we titrated the amount of anti-CD3 and anti-CD28 antibodies with the range concentration of 1-5 µg/ml (see *Table. 3.2*).

Table 3.2: Comparison of different anti-CD3/CD28 concentrations to optimize cell activation. *$CD8^+$ T cells were stimulated with different concentrations of plate-bound anti-CD3 and soluble anti-CD28 antibodies for 17 hours prior to flow cytometry measurements of the cell surface expression of CD69.The concentrations of anti-CD3 are labelled in black, whereas the concentrations of anti-CD28 are written in red. % illustrates the amount of cells that have CD69 in their surface.*

	1	**2**	**3**	**4**
A	5 µg/ml /5 µg/ml 81%	2 µg/ml /5 µg/ml 49%	1 µg/ml /5 µg/ml 41%	0.5 µg/ml /5 µg/ml 13%
B	5 µg/ml /2 µg/ml **87%**	2 µg/ml /2 µg/ml 52%	1 µg/ml /2 µg/ml 43%	0.5 µg/ml /2 µg/ml 33%
C	5 µg/ml /1 µg/ml 85%	2 µg/ml /1 µg/ml 47%	1 µg/ml /1 µg/ml 42%	0.5 µg/ml /1 µg/ml 12%
D	5 µg/ml /0.5 µg/ml 84%	2 µg/ml /0.5 µg/ml 50%	1 µg/ml /0.5 µg/ml 21%	0.5 µg/ml /0.5 µg/ml 7%

After $CD8^+$ T cells were stimulated for 17 hours using the concentration of anti-CD3/28 mentioned in *figure 3.2*, flow cytometry was used to confirm their activation response by measuring the expression of cell surface activation marker CD69. It was found that the highest level of activated cells indicated as $CD69^+$ cells was achieved using 5 µg/ml of plate bound anti-CD3 and 2 µg/ml soluble anti-CD28, resulting in 87% $CD69^+$ cells (see *Figure 3.2*). This concentration was used for T cell activation throughout the thesis.

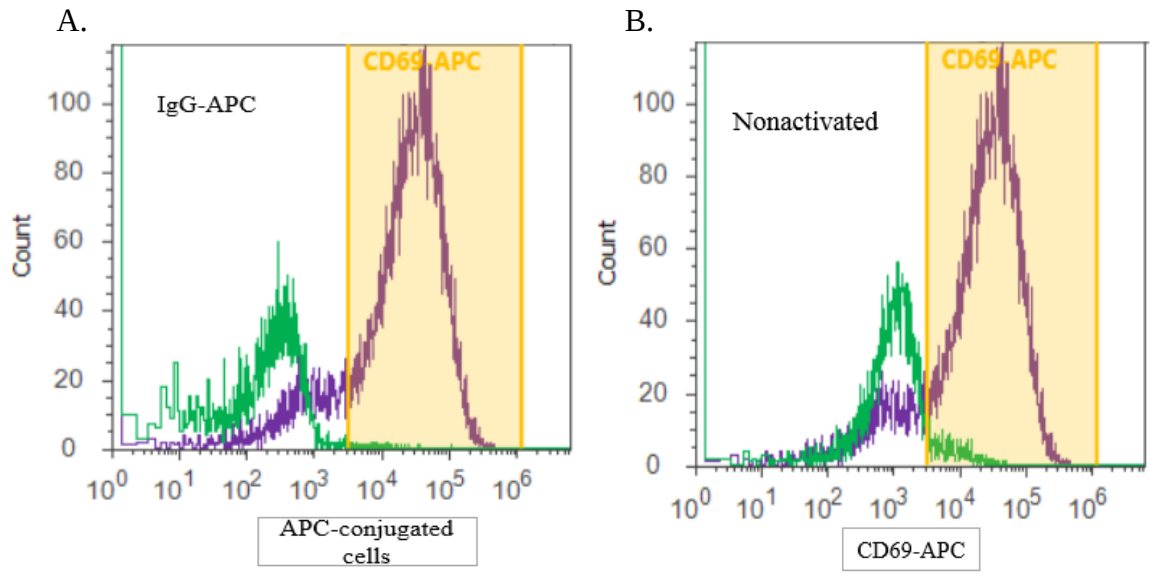


Figure 3.2: CD69 expression in $CD8^+$ T cell activated with 5 µg/ml of plate bound anti-CD3 and 2 µg/ml soluble anti-CD28. *$CD8^+$ T cells were stained before and after activation with APC-conjugated anti-CD69 antibody for measuring cell surface expression of CD69, an IgG-APC isotype control was also indicated.* ***A.*** *Green line, shows staining by the IgG antibody, staining 1% of the cells, whereas the purple line shows the staining of CD69-APC on activated $CD8^+$ T cells. In this case, 87% are $CD69^+$.* ***B.*** *Green line shows the staining of nonactivated cells with CD69-APC (9% were $CD69^+$), while purple line illustrates the activated $CD8^+$ T cells stained with CD69-APC (as the purple line in A, 87% are $CD69^+$).*

3.3 Cell viability before and after stimulation cells activated with antibody coated plate versus antibody covered Dynabeads.

Before we went on to study VDR expression in activated $CD8^+$T cells, we wanted to examine the cell viability among $CD8^+$T cells activated with plate bound antibodies and Dynabead covered beads. Even though antibody coated plate and antibody covered Dynabeads are the two methods used for T cell activation (52, 55, 73), we wanted to examine which activation method leads to high cell death. Other students in the lab have experienced cell death when stimulating with anti-CD3/CD28 covered beads (personal communication). But is this also the case for $CD8^+$T cells? To answer this question, we proceed with the activation of freshly isolated $CD8^+$T cells (from one donor) for 17 hours using:

1) 5µg/ml of plate bound anti-CD3 and 2µg/ml soluble anti-CD28 and 2) 1:4 bead to cell ratio of anti-CD3/CD28 antibodies covered beads.

In order to verify the cell viability, the percentage of live cells were measured by an automated cell counter (see section 2.2) before and after stimulation as viewed in the following *Table 3.3.*

Table 3.3: Percentage of live $CD8^+$T cells (isolated from the same donor). *The table illustrates measured live cells before and after stimulation activated with either plate bound antibodies (experiment number 1-3) or 1:4 Dynabead bound antibody (experiment number 4-6). 94% of freshly isolated $CD8^+$T cells were live cells.*

Experiment	% Live cells before stimulation	% Live cells after stimulation	Methods used to active cells
1	94	79	5µg/ml of plate bound anti-CD3 and 2µg/ml soluble anti-CD28
2	94	85	
3	94	81	
4	94	84	Dynabeads activated cells (1:4) bead to cell ratio
5	94	88	
6	94	89	

After measuring cell live before and after cell stimulation, no variation is seen among plate bound activation and Dynabead bound activation (*p=0.08*) (see *appendix 4, Table 5.5*) suggesting that both activation methods do not lead to major cell death and are thus, adequate activation methods for $CD8^+$T cells.

3.4 Expression of Vitamin D Receptor in $CD8^+$ T cells

It has previously observed that VDR expression is upregulated upon $CD4^+$ T cells activation (52). We wanted to see if this case relies the same in $CD8^+$ T cells. The timepoint when the expression of VDR is the highest must be known prior to vitamin D addition. To do so, isolated $CD8^+$T cells from four healthy donors were stimulated using 5 µg/ml plate bound anti-CD3 and 2 µg/ml soluble anti-CD28 antibodies for 17 and 40 hours respectively. *Figure 3.3 A.* shows the expression of *VDR* relative to PGK1 using qPCR.

There is not always a correlation between mRNA and protein, so we wanted to verify whether we can observe VDR expression at the protein as well. Therefore, further tests were carried out with Western blot as shown in *Figure 3.3 B.* The quantity of VDR was compared to the relative protein, Actin, which also serves as a loading control.

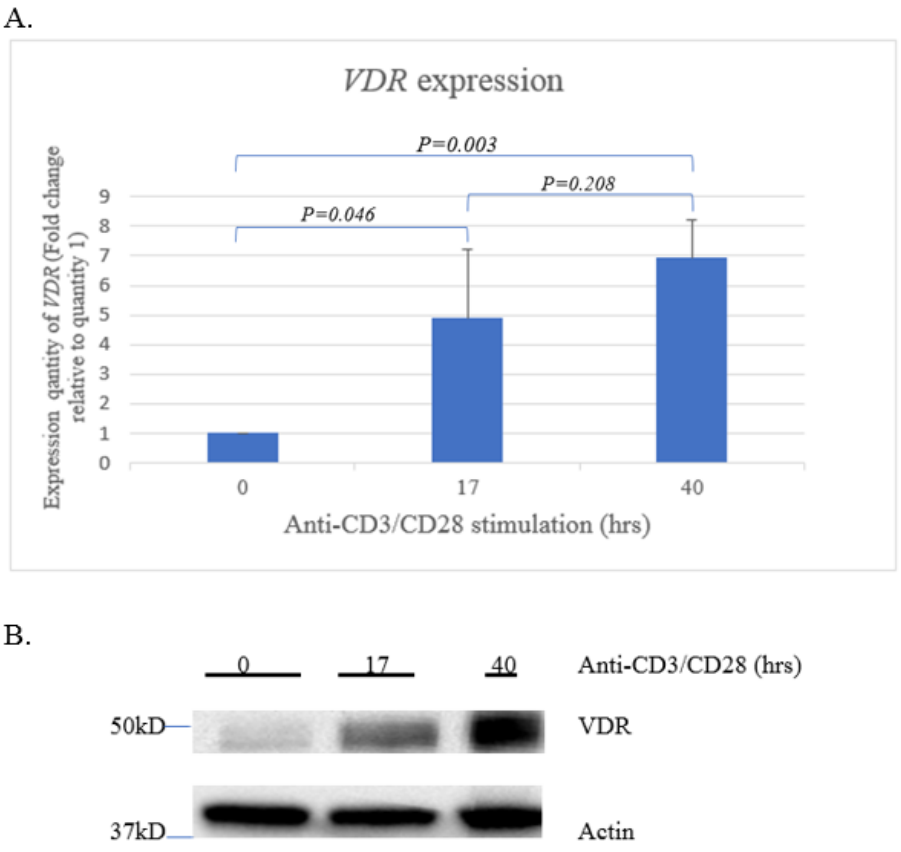


Figure 3.3: VDR expression is the highest after 40 hours of anti-CD3/CD28 stimulation shown by both qPCR and Western blotting. A. *Bar chart showing fold change in VDR gene expression, including error bar representing standard errors of each hours of stimulation from four donors. Values represents fold change relative to nonactivated cells (time point 0). Paired student's t-test was performed in VDR expression comparing before (0 hr) and after 17 hrs of stimulation (p=0.046), between 17 and 40 hrs of stimulation (p=0.208) and between 0 and 40 hrs of stimulation (p=0.003) (see appendix 4, Table 5.1 for statistics). p<0.05 is considered significant.* ***B.*** *Protein of cell lysates from nonactivated cells and cells stimulated for 17 and 40 hours were separated by SDS-PAGE prior to Western blot using anti-VDR and anti-Actin (loading control) antibodies for detection. This immunoblot is one experiment (D1) out of two experiments performed.*

As shown in *Figure 3.3 A*, VDR expression is statistically significant among 0 (non-stimulated) and 40 hours of cell stimulation giving *p=0.003* compared to 0 to 17 hours and 17 to 40 hours of stimulation. Line graph in *appendix 5* illustrates the change of *VDR* expression from non-stimulated (0 hour) to 17 and 40 hours of stimulation in each donor. Thus, VDR expression at both mRNA and protein level was confirmed to be at highest level after 40 hours of cell stimulation. Therefore, this was further chosen as the time point where vitamin D was added in the next experiments.

3.5 Active form of vitamin D (calcitriol) affects the expression of *VDR*, *TAGAP* and *CYP24A1* in $CD8^+$ T cells.

In the current thesis we wanted to evaluate whether $CD8^+$ T cells (isolated from three healthy donors) showed vitamin D responsiveness by adding 10 mM calcitriol to the cell culture 40 hours after cell activation. Other study (52) have shown that TAGAP expression is reduced and CYP24A1 is increased upon vitamin D treatment. Therefore, we wanted to see if these genes responded in a similar manner after calcitriol addition of $CD8^+$ T cells. First, relative expression of VDR (Figure 3.4) was measured to ensure that VDR is highly induced after 40 hours of anti-CD3 and soluble anti-CD28 antibodies stimulation.

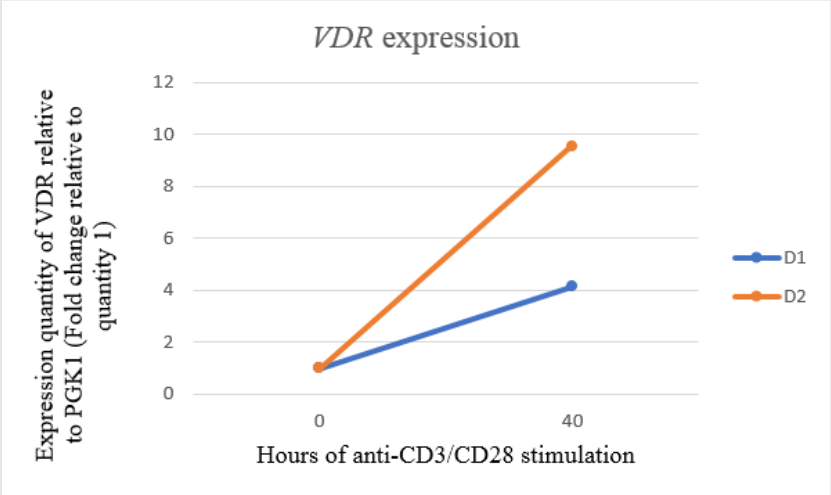


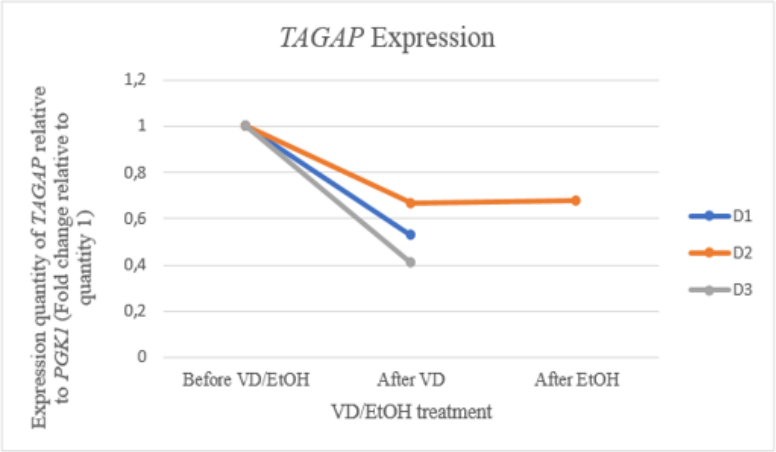
Figure 3.4: The expression of VDR before (0 hr) and after (40 hrs) anti-CD3/CD28 stimulation. *VDR expression is highly induced after 40 hours of stimulation in two donors, D and D2, D3 (missing data).*

After T cell activation for 40 hours, cells were treated with 10 nM calcitriol or vehicle control for three hours prior to cell harvesting. Due to limited number of $CD8^+$ T cells from two out of three donors, only cells from one donor (D2) were also treated with vehicle control (ethanol). *CYP24A1* is a well-established vitamin D responsive gene and is known to be upregulated upon vitamin D treatment, whereas *TAGAP* has been shown to be down regulated in $CD4^+$ T cells

after vitamin D treatment (52). We wanted to find out if these genes were also affected in $CD8^+$ T cells after vitamin D addition and can be used as positive controls for vitamin D response.

After treatment with vitamin D or vehicle control for three hours, RNA was purified from the cells and used to measure *TAGAP* and *CYP24A1* gene expression by qPCR relative to the *PGK1* reference gene. Relative expression of *TAGAP* (*Figure. 3.5 A.*) is downregulated after vitamin D treatment. The downregulation is indicated in all three donors. However, as the same downregulation is observed also in the ethanol treated cells, this is probably not mediated by vitamin D. Relative expression of *CYP24A1* (*Figure. 3.5 B.*) is upregulated after vitamin D treatment in two donors, but surprisingly it shows slightly downregulation in the third donor (D2) after both vitamin D and ethanol treatment. We can conclude that data from two out of three donors indicate upregulation of CYP24A1 expression, however, whether this is a specific effect on vitamin D treatment cannot be stated as no vehicle control was included from these two donors.

A.



B.

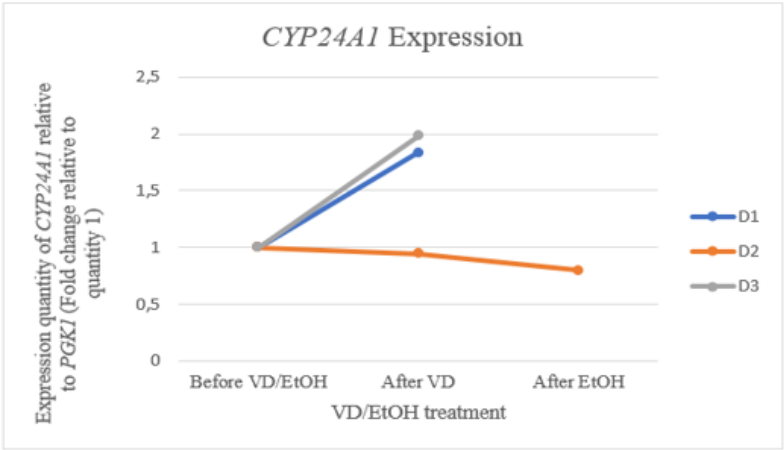


Figure 3.5: Expression of TAGAP and CYP24A1 in activated CD8$^+$ T cells after vitamin D treatment. *The relative mRNA expression of **A**. TAGAP and **B**. CYP24A1 from three donors before and after vitamin D (VD) of activated CD8$^+$ T cells. A vehicle control (i.e. ethanol, EtOH) is included for one of the donors. Gene expression was quantified relative to PGK1 expression. Values represents fold change relative to before VD/EtOH treatment. Paired student's T-test was performed to compare TAGAP expression ($p=0.025$) before and after vitamin D treatment (see appendix 4, Table 5.4). $p<0.05$ is considered significant. We decided not to perform Paired t-test for the expression of CYP24A1, since we observed slight downregulation in D2 donor.*

3.6 Optimizing VDR-ChIP procedure

After verifying that cells were responsive to vitamin D, they were further treated with either anti-VDR or isotype (IgG) control antibodies covered protein A Dynabeads. Before, proceeding with this cells, VDR-ChIP procedure including crosslinking, lysis buffer and sonication protocols must be optimized. In this project, ChIP is a method used to precipitate DNA bound by VDR, using specific anti-VDR antibodies. The precipitated DNA can be analysed by sequencing, real-time PCR or by PCR using specific designed primers. The MS research group aims to develop a ChIP-seq pipeline, however, in the current master thesis project, we aim to

perform the initial DNA analyses with PCR. The ChIP protocol includes many steps which are visualized in *Figure 3.6*.

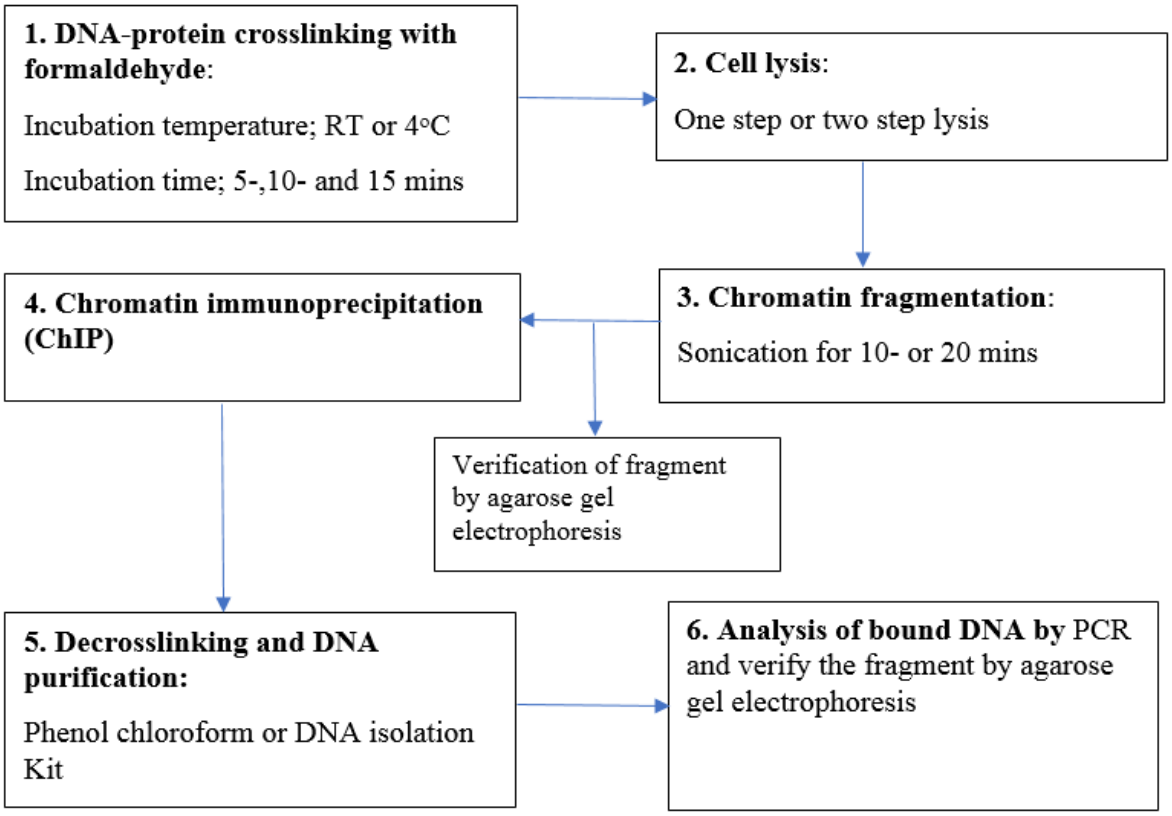


Figure 3.6: Different steps of the ChIP protocol. *The flow chart illustrates the steps of the ChIP protocol and the tests that were done within each step in the current thesis.*

- 3.6.1 Optimization of procedures for crosslinking and sonication

DNA/chromatin needs to reach an average length of 100-300 base pairs, as evaluated by agarose electrophoresis. Chromatin is fragmented using a sonicator. Prior to sonication, the cells need to be fixed in order to cross-link DNA and protein, following by cell lysis and sonication. The fixative, the length and temperature used during fixation as well as the cell lysis protocol and the length and strength of sonication affect chromatin fragmentation (74). The protocols differ between different cells and tissues and they need to be optimized for each material used. In this thesis, we aim to do ChIP in $CD8^+$ T cells. However, to achieve sufficient number of cells for testing the described conditions, we started to evaluate the protocols using PBMCs. The optimal protocol for PBMC was thereafter confirmed in purified $CD8^+$ T cells.

5-10 million PBMCs were fixed with formaldehyde for 5-, 10- and 15 mins at 4°C or at RT. Cells were lysed as described in section 2.8.2. following sonication for either 10 min (30 sec

on/off, 10 cycles) or 20 min (30 sec on/off, 20 cycles). Sonicated chromatin was isolated using phenol-chloroform extraction as described in section 2.8.3 and analysed by agarose gel electrophoresis.

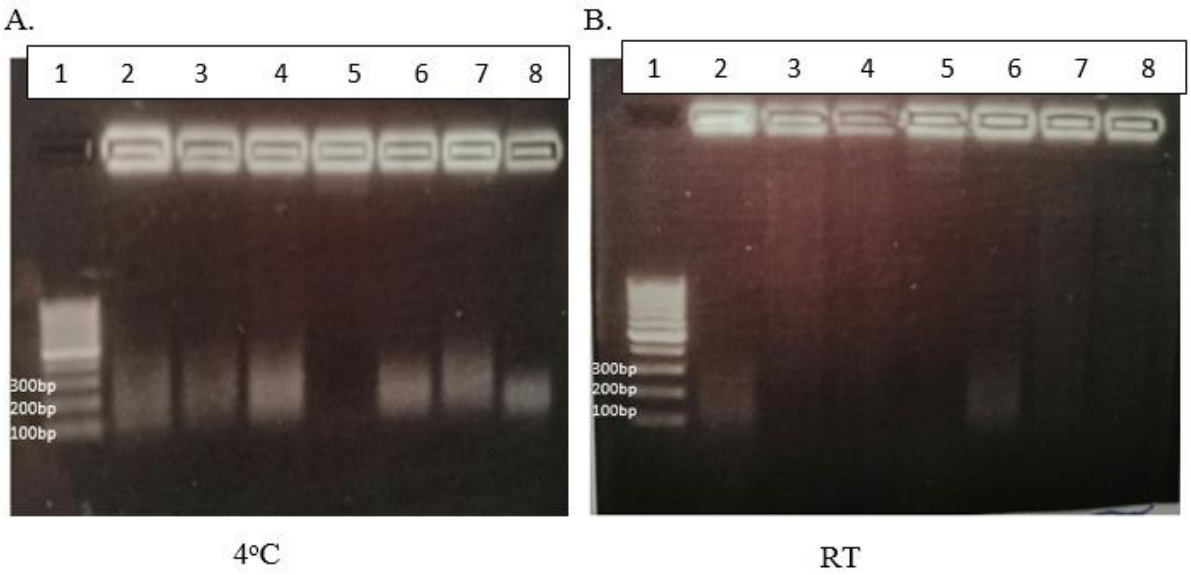


Figure 3.7: Optimization of crosslinking and chromatin fragmentation*. Agarose gel electrophoresis of DNA from PBMCs crosslinked at 4°C in* ***A.*** *and at RT in* ***B.*** *Well nr. 1 in both figures are 100bp gene ruler. Well nr. 2- 4 samples from cells that were fixed for 5, 10 and 15 mins respectively and sonicated for 10 mins. Well nr. 6, 7 and 8: samples from cells that were fixed for 5, 10 and 15 mins and sonicated for 20 mins. Well nr. 5 is negative control, which is fixed sample that were not subjected to sonication.*

In well number 4 in *Figure 3.7 A*, the length of fragmented DNA is 100-300 bp and there is much more input (fragmented DNA) compared to the other fragmentation, suggesting that it is better for fix cells at 4°C for 15 min and the length of the sonication does not seem to have any impact on the fragmentation of the DNA isolated from cell fixed in 4°C.

We observed strong bright band at a high molecule weight in all the wells of each agarose gel experiment. This is something that should not be there. To clarify the reason behind this, DNA was purified using a classical purification step (Phenol-chloroform) and a DNA column kit. The DNA was purified using DNA isolation column kit, does not have the high molecular band in the wells, as it is shown in *Figure. 3.7*.

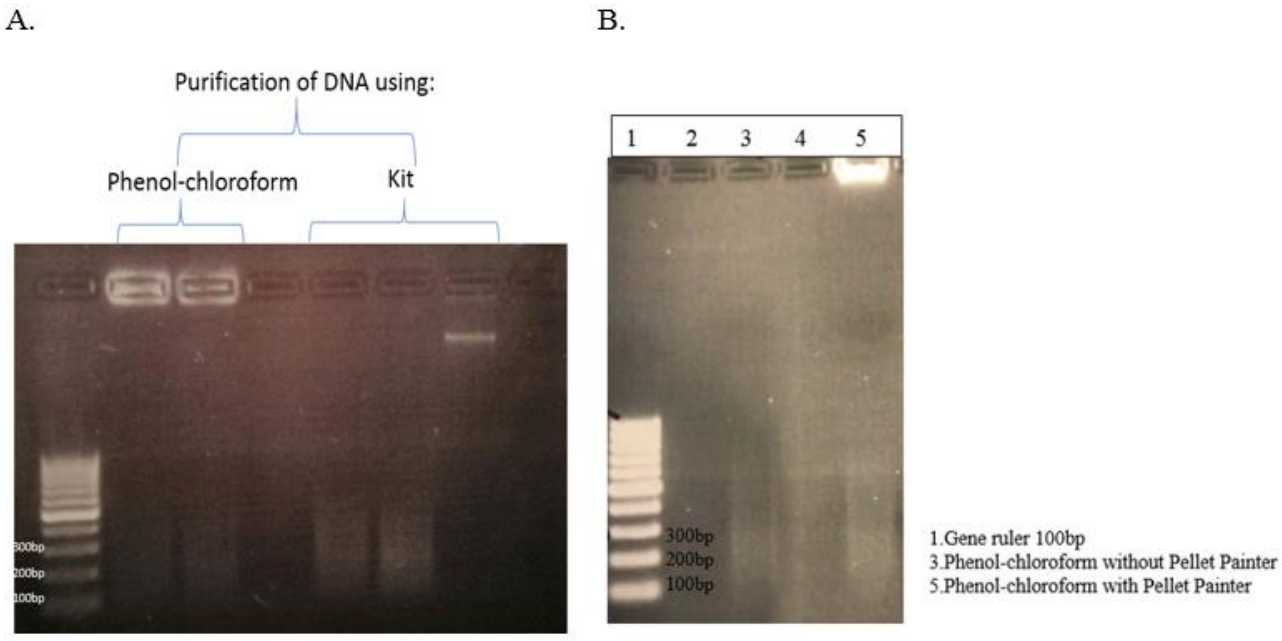


Figure 3.8: Analysing the size of fragmented in agarose gel electrophoresis. ***A***. *Agarose gel of purified DNA (from PBMCs that were disrupted by one step lysis) using Phenol-chloroform method and DNA isolation column kit. Negative control (non-sonicated) DNA was included in the upper right side of gel in A, showing that it has barely migrated.* ***B***. *Agarose gel of purified DNA from PBMC investigating purification of DNA using phenol-chloroform with (well nr 5) and without pellet painter (well nr.3). Bright band was observed in the well nr. 5 of DNA purified using phenol-chloroform with pellet painter.*

This led us to suspected that the DNA paint is the reagent resulting in this high molecular band in the wells. Therefore, we performed another gel electrophoresis to compare cells purified using phenol-chloroform with and without cell pellet painter in *Figure 3.8.B*. No band is observed in the cells purified phenol-chloroform without pellet painter. We have also observed that we purified more DNA fragments after using DNA purification kit (see *Figure 3.8 A*.) compared to DNA purified using phenol-chloroform.

- 3.6.2 one step versus two lysis buffer

In parallel with testing crosslinking and sonication parameters, we also wanted to test two different lysis buffers; one-step (cell lysis) and two-step (cell and nuclear lysis). VDR is a receptor that is found in cytosol in cells that are not treated with vitamin D, whereas in cells that are vitamin D treated, the receptor goes in the nucleus and regulate transcription of its target

genes. In this case, we are only interesed on VDR that are inside the nucleus. As mentioned in section 2.8.2, PBMC were lysed using these two lysis bufferes (see *Figure 3.9*).

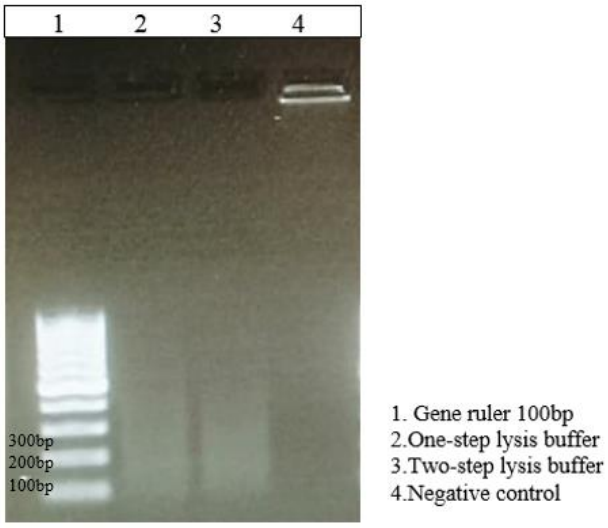


Figure 3.9: PBMC that were lysed using one and two step lysis buffers. *The amount of fragmented DNA isolated from cells is the same in both lysis buffers.*

No-variation was observed in the level of fragmented DNA isolated from cells that were lysed using either of the buffers. Although, no variation was seen among the lysis buffers, we decided to use two-step lysis in the downstrem expermiments since we are just interessed on investigating VDR content from mucleus. After verifying that the pellet painter is the one causing the band in the well and testing lysi buffers in PBMC , we wanted to test the same conditions in $CD8^+$ T cells. $CD8^+$ T cells were crosslinked at 4^oC for 15 min, lysed using two-step lysis buffer and sonicated for 10 min. The DNA was further purified using phenol-chloroform without pellet painter and the result is viewed in *Figure 3.10*.

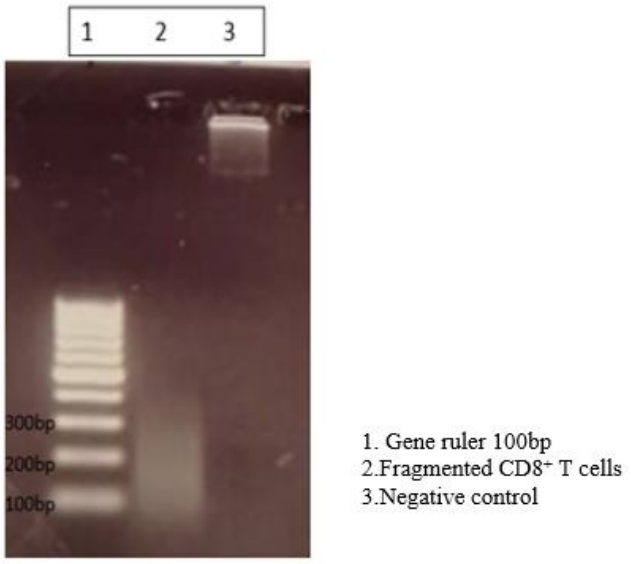


Figure 3.10: Agarose gel of chromatin from CD8$^+$ T cells. *The cells were disrupted by two step lysis, with the fragments of the optimal size range; 100-300 bp, including a negative control that have barely migrated.*

All the cell conditions; cell activation, crosslink, lysis and sonication are by now optimized in CD8$^+$ T cells and we would like to proceed with ChIP procedure.

3.7 Immunoprecipitation of DNA regions that are bound by VDR

Based on a ChIP study done in CD4$^+$ T cell by Handel *at al*., we chose to analyse selected DNA fragments that are bound VDR in CD4$^+$ T cells (69) to analyse whether these DNA fragments are occupied by VDR also in CD8$^+$ T cells. This is not known and was tested in the current thesis.

After optimization of chromatin fragmentation and confirming CD8$^+$ T cell's response to vitamin D after cell activation (see Figure 3.5), VDR ChIP following PCR was performed in D2 and D3 in order to identify the DNA region bound by VDR. Cells from D1 were treated only with vitamin D, whereas cells from D2 were treated with either vitamin D or EtOH. All chromatin except for 100 µl (designed as 'Input') was used in the ChIP experiments. DNA was purified from the remaining chromatin preparation. VDR specific antibodies were used to pull down the genes that were bound by VDR. Isotype (IgG) control was included and served as a negative control. Four primer pairs were used for amplification of regions previously shown to be bound by VDR in CD4$^+$ T cells, covering regions in the *MYC, TAGAP (*two different regions*)* and *VDR* genes. DNA from the 'Input' samples (one per ChIP) as well the ChIP samples was purified, and PCR was performed to determine whether these genes were precipitated during the ChIP protocol. Input DNA were diluted to 8, 2 and 0.5 ng and were used in the PCR

reactions. This was to compare the strength of the band from the DNA pulled down after VDR or IgG ChIP.

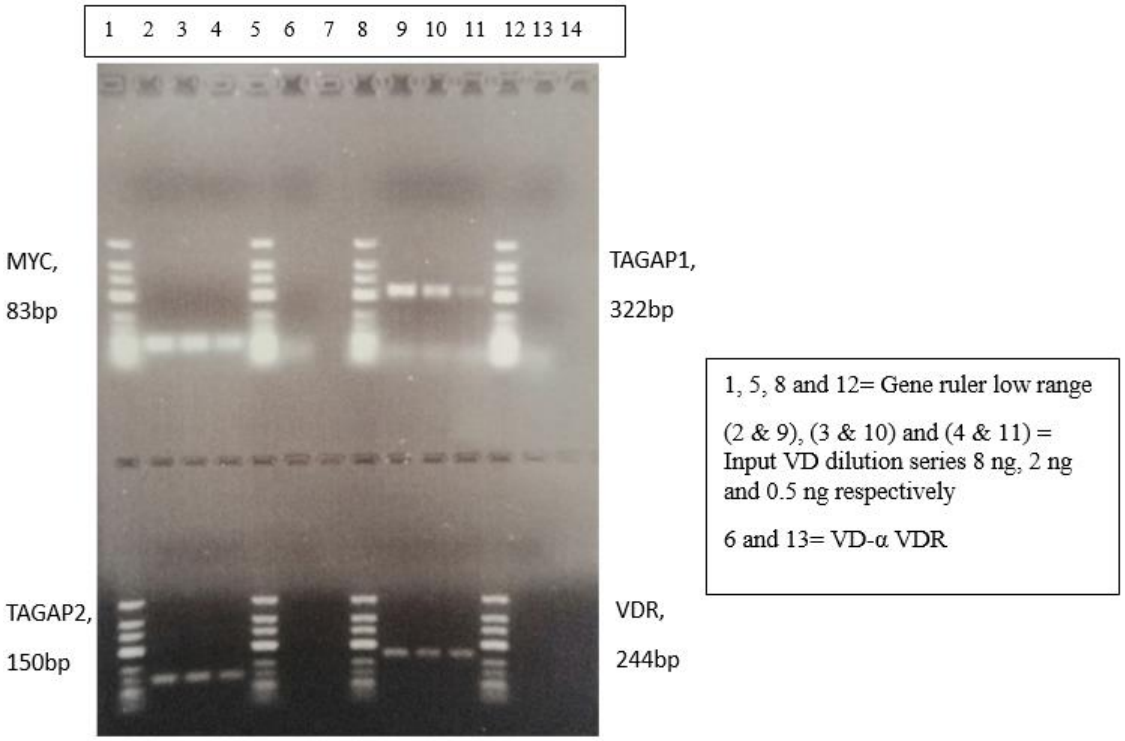


Figure 3.11: PCR amplification of immunoprecipitated DNA from CD8$^+$ T cells isolated from D1. *CD8$^+$ T cells purified from D1 was stimulated with anti-CD3/CD28 antibodies prior to treatment with vitamin D (VD). PCR were run on serial dilutions of the input DNA using 8, 2 and 0.5 ng as input. All four primer pairs (as indicated in the figure) were used for the input samples as well as for the immunoprecipitated DNA. IgG control was not included, and no band were indicated in the samples, suggesting that all four DNA regions were not bound by VDR.*

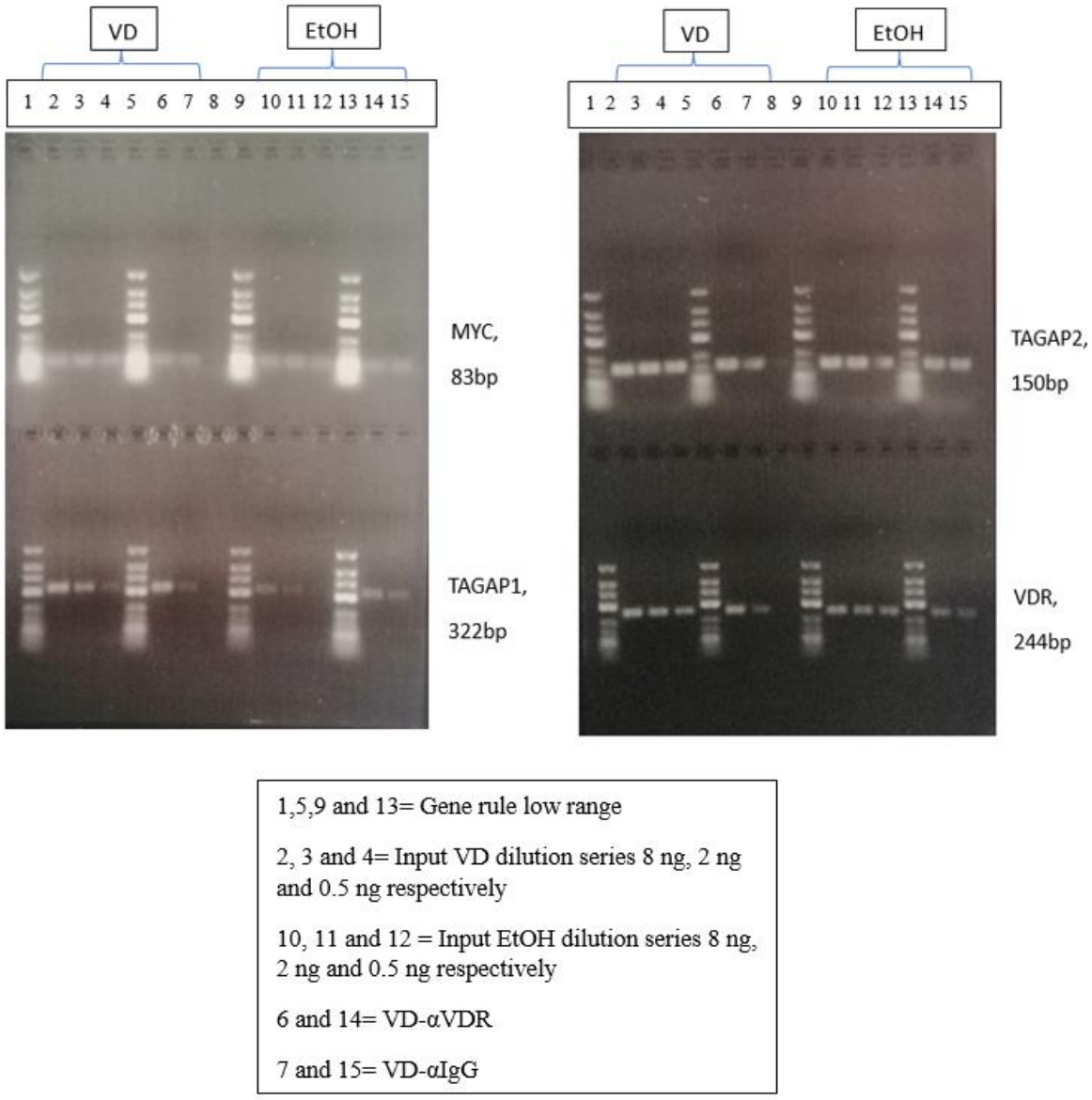


Figure 3.12: PCR amplification of immunoprecipitated DNA from cells isolated from D2*. CD8⁺ T cells purified from D2 were stimulated with anti-CD3/CD28 antibodies prior to treatment with vitamin D (VD). PCR were run on serial dilutions of the input DNA and EtOH using 8, 2 and 0.5 ng as input. All four primer pairs (as indicated in the figure) were used for the input samples as well as for the immunoprecipitated DNA. IgG control was included in this analysis. Bands were indicated in both vitamin D and EtOH samples, suggesting that all four DNA regions were bound by VDR. Weak bands were also indicated in IgG controls (in both vitamin D and EtOH samples).*

After performing VDR ChIP of samples from the two donors, DNA was purified, and PCR performed. Strong signals from all four potential VDR binding sequences were observed in samples after VDR ChIP from D2, see *Figure 3.12*, both in ethanol and vitamin D treated samples. However, we did not observe any PCR products from the DNA purified after VDR ChIP in D1, see *Figure 3.11*. Surprisingly, the DNA purified in the IgG isotype controls samples were also positive for the four PCR reactions in samples from D2. Thus, to analyse whether the

VDR or IgG ChIP, pulled down sequences that are also not expected to interact with VDR, another PCR reaction with primers against a region that is previously shown to be bound by VDR in T cells (69), serving a negative control for ChIP was also included. The gene region chosen for this test was an intronic region from *GAPDH*. The result is illustrated in *Figure. 3.13*. However, *GAPDH* came out positive for both donors in cells treated with vitamin D and ethanol.

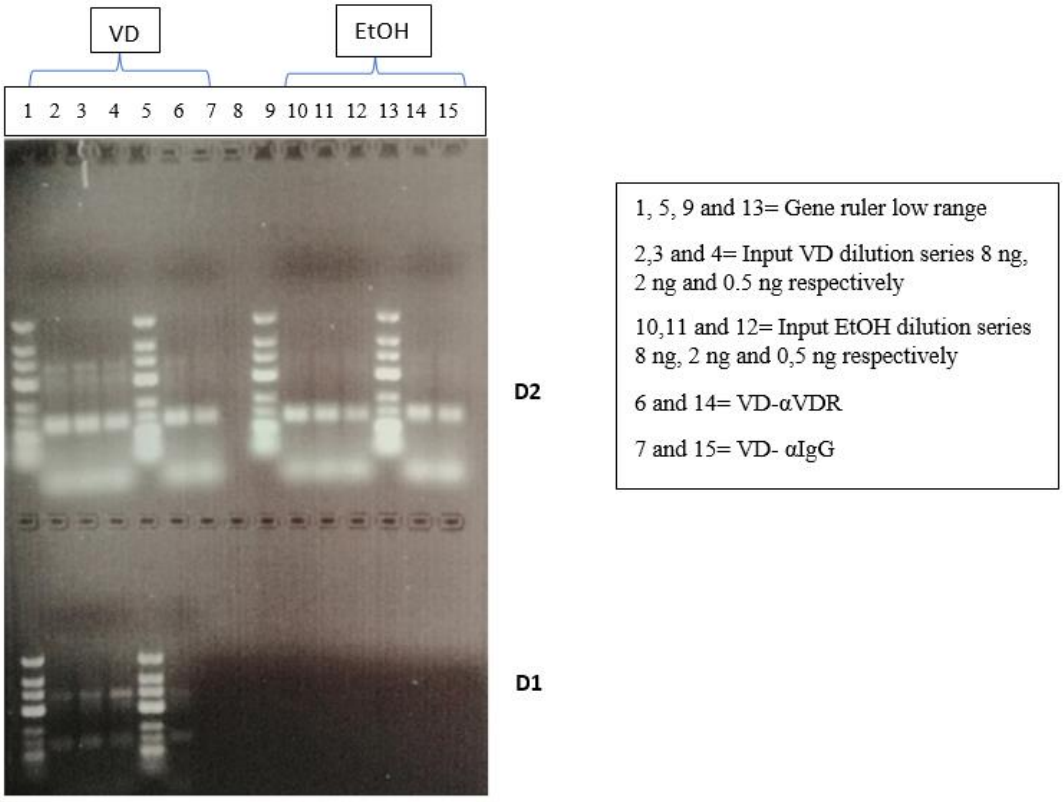


Figure 3.13: Displays the precipitated GAPDH Intronic 2 in both D1 and D2*. PCR were run on serial dilutions of the input DNA and EtOH using 8, 2 and 0.5 ng as input. Bands was observed in all samples and IgG controls treated with either vitamin D (VD) or EtOH.*

4 Discussion

T cells are important players in the pathogenesis of MS. Although $CD8^+$ T cell are more abundant in MS lesions compared to $CD4^+$ T cell (4), the role of $CD8^+$ T cell in MS is still not clear. Low level of vitamin D is shown to be involved in the progression of MS (31). However, the mechanisms behind increased MS risk and low serum vitamin D levels are poorly understood. To gain more knowledge about the relation of $CD8^+$ T cells, vitamin D and MS genetic risk factors, we wanted to establish a VDR-ChIP protocol in $CD8^+$ T cells in order to identify new DNA binding site for VDR. This chapter includes the discussion of the results following methodological contemplations.

4.1 Why does cell purity vary among donors?

To ensure that the isolated cells were indeed $CD8^+$ T cells, the purity of $CD8^+$ T cells was confirmed by flow cytometry by staining cells with FITC conjugated mouse anti-human CD8 (HIT8α monoclonal antibody). This antibody specifically binds to CD8α, which are expressed by $CD8^+$T cells. Of note, natural killer (NK) cells also express low levels of CD8 receptor. As seen in *Table 3.1*, the purity of isolated $CD8^+$ T cells varied among the donors. This variation might be because different proportions of NK cells are present in different donors, and that these $CD8^{low}$ cells are not captured in the gate for $CD8^+$ T cells. In addition, NK cells expressing the CD8 receptor might thus appear in the gate we name $CD8^+$ T cells and be considered as $CD8^+$ T cells even if they are not. By staining the cell population with antibodies specific to NKG2D, a NK surface receptor (activation marker) that is upregulated upon NK cell activation (75), is one way to control for the presence of NK cells in the $CD8^+$ T cell population.

4.2 Which activation method is adequate to use: antibody covered Dynabeads or antibody coated plate?

Stimulating cells using anti-CD3/CD28 covered beads may cause lower degree of variation across experiments compared to stimulating cells with plate bound antibodies. This is because the beads have a fixed amount of antibodies on the surface (made commercially), while plate bound antibody solution is prepared by us by diluting the stock concentration of the antibodies to 5 and 2 µg/ml before each individual experiment, and are thus more prone to variations between the experiments. We investigated the variance of cell activation in our experiment with both antibody coated plate and antibody covered Dynabeads (data not shown). Plate bound activation gave high proportion of activated cells at each experiment (varying from 80-84%). Unfortunately, we could not measure the degree of activation of $CD69^+$ $CD8^+$ T cells in cells

activated with Dynabeads by flow cytometry, but rather with qPCR to measure the mRNA expression of CD69. As anti-CD3/CD28 covered Dynabeads are big in size, they must be removed prior to flow cytometry. Since $CD8^+$ T cells were isolated using positive selection (beads are directly bound to $CD8^+$ T cells) from whole blood as described in section 2.1.2, beads remain bound to the cells after purification and cannot be removed.

Furthermore, it was not possible to compare the variance between the two activation experiments as the same activation methods were not used. In addition, flow cytometry measured $CD69^+$ per cell, whereas qPCR measured the expression of CD69. By performing qPCR for plate bound activated cell, the variance between the two experiments could be compared. However, we measured cell viability before and after cell stimulation to verify the activation method that gave the lowest cell death.

4.3 Cell viability before and after cell stimulation

Previous experiments from the lab have shown that 1:1 bead to cell ratio gave high degree of dead cells (personal communication) after activation. This is consistent with the result we got (data not shown), resulting in the high degree of cell death after stimulation (94-60%), whereas 1:4 bead to cell ratio led to low cell death (94-92%) without affecting the activation response. Therefore, 1:4 bead to cell was used to stimulate $CD8^+$ T cells. As illustrated in *Table 3.3*, the percentage of live cells after stimulation was reduced compared to before stimulation. This finding is consistent to what has been observed earlier (76). These naïve cells are activated in an environment that is different from their natural environment. Cell variability was almost the same using both techniques as viewed in *Table 3.3*.

Performing qPCR to confirm cell activation is time consuming and requires more cells to be analysed compared to flow cytometry. Antibody coated plate is more gentle activation method that goes well with activation response of $CD8^+$ T cells and led to better viability. Therefore, this activation method is more adequate and suitable for the stimulation of positive selected isolated $CD8^+$ T cells and is chosen to be used for the downstream activation experiments.

4.4 $CD8^+$ T cell activation using antibody coated plate to induce VDR expression

In naïve T cells, the expression of VDR is generally low and is induced by activation through the TCR (29). In order for the cell to have sufficient amounts of VDR upon vitamin D treatment, we titrated the amount of anti-CD3/CD28 antibodies for activation (see section 3.2) to investigate which concentration gave the highest frequency of activated $CD8^+$ T cells (measured by CD69 cell surface expression). The highest level of activation was achieved using 5 µg/ml

plate bound anti-CD3 and 2 µg/ml soluble anti-CD28 antibodies, resulting in 87% activation. There are many other protocols with different concentration of ani-CD3/CD28 used for T cell activation, but the concentration that is used in this thesis is comparable with study done by Forster *at el* (73).

VDR was observed to be significantly increased after 40 hours of stimulation compared non-stimulated (0 hours) and after 17 hours of stimulation (see *appendix 4, Table 5.1*). Thus, cells were stimulated for 40 hours prior to vitamin D addition.

4.5 Vitamin D responsive genes; *TAGAP* and *CYP24A1*

The level of vitamin D can vary among donors. In addition, the activation status of the purified cells varies and might cause different results for VDR expression at baseline. However, after T cell activation, VDR expression was induced in all experiments. When VDR expression reached a high level, the cells were treated with 10 nM active vitamin D and EtOH (vehicle control) and expression of selected vitamin D response genes were analysed by qPCR. Due to limited numbers of $CD8^+$T cells, only cells from D2 were treated with vitamin D and EtOH, while cells from D1 and D3 were treated with vitamin D only. 10 nM calcitriol is frequent used in vitro in other studies (52). Vitamin D was added directly to the cell suspension, which may cause high local concentration directly to the cells and induce more regulation of target genes in certain cells compared to physiological conditions.

CYP24A1 and *TAGAP* were regulated by vitamin D in $CD4^+$ T cells (52). The downregulation of *TAGAP (p=0.025) (see appendix 4, Table 5.4)* was reduced more than 0,5-fold after 3 hours of vitamin D treatment, showing that this is likely regulated directly from VDR itself and not through other transcriptional factors that regulates VDR. At the time of vitamin D addition, the cells were transferred from their original activation plate (coated with anti-CD3) into new activation plate (not coated with antibodies). Antibodies for the activation of cells were not added to the plate as these cells were treated with vitamin D for three hours, therefore, the slight downregulation of *CYP24A1* (in vitamin D and EtOH treated cells form D2) and *TAGAP* (in EtOH treated cells from D2), was probably not mediated by vitamin D, it could simply be that the cells were no longer activated. We should have added anti-CD3/CD28 Dynabeads in parallel to vitamin D addition to further stimulate the cells and follow how the expression of *TAGAP* and *CYP24A1* change.

Our results in $CD8^+$ T cells are in agreement with previous studies showing that *CYP24A1* is upregulated in activated $CD4^+$ T cells upon vitamin D addition (52). The role of *CYP24A1* is to

deactivate the active vitamin D and the level of *CYP24A1* is proportional with the amount of active vitamin D. The upregulation of *CYP24A1* is seen in two of three donors (D1 and D3), while downregulation was seen in the third donor (D2). Since the expression of *CYP24A1* and *TAGAP* genes are induced upon activation of $CD8^+$ T cells, they can be used as a positive control for vitamin D response. Our results from $CD8^+$ T cells showing downregulation of *TAGAP* expression and upregulation of *CYP24A1* expression after vitamin D treatment is in line with what is observed in $CD4^+$T cells (52). The findings indicate that the gene regulation was induced with vitamin D addition, but since more EtOH control was not included, it is difficult to conclude and that further studies are required in order to confirm the findings.

4.6 Methodological contemplations

To our knowledge, a procedure on VDR-CHIP in $CD8^+$ T cells has not been performed previously. Thus, the aim of this thesis was to optimize a protocol for VDR-ChIP in $CD8^+$ T cells in order to identify new binding sites for VDR.

• 4.6.1 Cross-linking, cell lysis and sonication optimization

Crosslinking is a step in the ChIP procedure that plays a major role in obtaining a successful sonication. It is important to sonicate DNA into smaller fragments, since very large fragments might contain several potential VDREs, in addition to none-relevant sequences, can make it difficult to identify the relevant DNA binding region. In this thesis, we use formaldehyde to crosslink DNA- bound protein. The reaction temperature (RT or 4^{o}C) and incubation time (5-, 10- and 15 min) during crosslinking was optimized. These optimizing parameters are dependent on the physical environment of the target protein, which varies between cytosolic and membrane proteins (77).

After crosslinking, we proceeded with cell lysis in order to free the cellular content, testing both one-step lysis and two-step lysis. As mentioned in section 1.7.4, choosing the right lysis buffer is essential for achieving the best possible yield. Since VDR is a nuclear receptor found both in the cytosol and nucleus, we wanted to confirm that it is nuclear VDR we are immunoprecipitating. The free VDR in the cytosol needs to be removed so they do not bind to specific VDR antibodies during the ChIP procedure. We found that the two-step lysis buffer gave almost the same chromatin yield after sonication compared to the one-step lysis. The first step in this procedure is cellular lysis, where the external membrane gets disrupted. The second step is nuclear lysis that disrupts the nuclear membrane and frees the nuclear content. In this way, we can access the VDR that is bound to target chromatin. However, due to time limitation, we were not able to test whether the two-step lysis resulted in more specific immune

precipitation compared to the one-step lysis. Sonication was tested for 10- and 20 min, as viewed in *Figure 3.7*. Sonicating the lysate for 10 and 20 min gave the same chromatin fragment in the expected range (300-100 bp), so, for further fragmentation of chromatin, we decided to sonicate the lysate for 10 min.

Furthermore, after ChIP, the DNA are purified from the precipitate. So, breaking the interaction between protein and DNA through a process called decrosslinking, is required. Proteinase K was used to degrade protein, while phenol-chloroform or DNA isolation kit were used to purify DNA. These two DNA purification methods were used to investigate what caused the signal/band viewed on the wells of agarose gel in *Figure 3.8* to appear. No-band was observed after purifying DNA using the kit, suggesting that were components within the phenol-chloroform protocol that was causing the high molecular band on the wells of the agarose gel. Pellet paint is composed of proteins that is precipitated together with the DNA in order to easily visualize DNA after centrifugation. This was added to the precipitated DNA in the last step of phenol-chloroform protocol. Later, we discovered that Pellet paint absorbs UV light, thus causing a bright, high molecule band as agarose gels were analysing. Once the Pellet Paint was removed from the protocol, this bright band disappeared as illustrated in *Figure 3.10*.

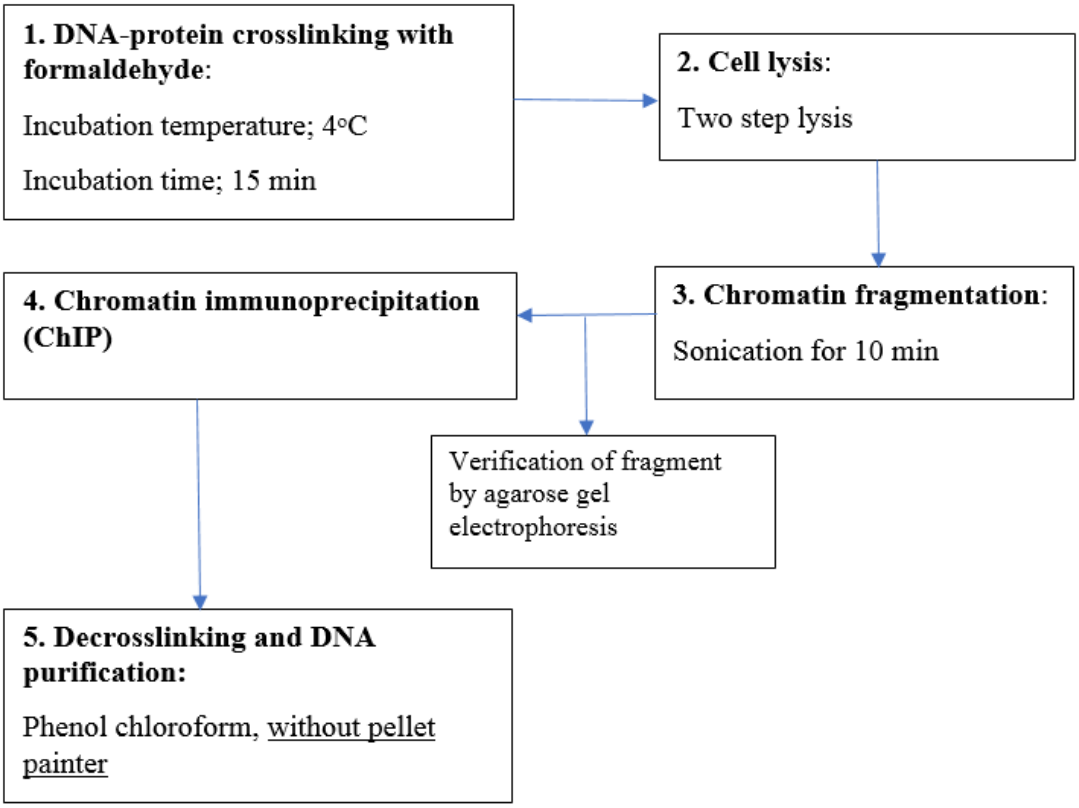


Figure 4.1: The pathway of optimized ChIP procedure. *This flow chart includes the parameters that gave best results within each step.*

4.7 Identification of precipitated DNA bound by VDR

After optimizing the cell activation and preparation of samples for ChIP, the entire VDR ChIP procedure was performed using an anti-VDR antibody in addition to an isotype control (anti-IgG). To confirm that the ChIP procedure was optimized, we chose DNA fragments (*MYC* and *TAGAP*) that are shown to be pulled down by VDR-immunoprecipitation in activated $CD4^+$ T cells (69) to investigate whether they could also be pulled down in $CD8^+$ T cells. After performing the ChIP procedure and PCR amplification of selected regions, immunoprecipitation of *MYC*, *TAGAP* and *VDR* was confirmed in one donor while no immunoprecipitation was observed in the second donor. By including a negative control, a region of DNA that does not bind VDR, we can verify unspecific binding of the VDR antibody. In addition to the isotype control (anti-IgG) and the vehicle control (EtOH), PCR using GAPDH primers served as a negative control for unspecific immunoprecipitated DNA was performed. We found non-specific bands in agarose gel after performing PCR, indicating positive results in the negative controls (IgG and EtOH) and in genes that were known to not be bound by VDR (GAPDH).

Some possible explanation for obtaining these results could be that protein A/G that are bacterial proteins that have the capacity to bind various immunoglobulins with different affinities. Protein A/G coated Dynabeads are antibody binding beads which help to capture DNA-Protein or protein-protein complexes. Since these Dynabeads can bind to various antibodies and if ChIP method is not fully optimized, these beads can non-specifically pull down some genes, which could be one of the reasons why GAPDH (negative control) were pulled down in both donors.

Another reason for the findings could be the monoclonal anti-VDR antibody. We decided to use this antibody as other researches in other lab use it to pull down VDR binding DNA regions (personal communication). Unfortunately, crosslinking can cover target epitope within the cells that monoclonal antibodies are specific to, and this could have led to negative reaction in D1. But since the ChIP procedure was only performed once, we cannot say that this antibody has caused the negative reaction. Thus, it might be wise to repeat the procedure using a polyclonal anti-VDR antibody that have the affinity to different epitopes within cells, which might increase in the immunoprecipitation of target DNAs.

An anti-IgG isotype control was included in the ChIP procedure to exclude signals from VDR-ChIP due to non-specific binding to the antibody's constant regions. Unfortunately, as illustrated in *Figure 3.12,* the anti-IgG control in D2 gave bands, suggesting that non-specific binding might have occurred and the results for this donor should not to be trusted. The reason for this could be incomplete washing after IP or contamination of input in the sample leading to pull down of other DNA regions that are not necessarily bound by VDR.

5 Conclusions and future perspective

In section 1, the theoretical and aim of the thesis was described following the methods used to investigate the aim were shown in section 2. The results were illustrated in section 3 and the discussion of these results were explained in section 4. In this final section, I will summarize the findings and some suggestions for future perspectives.

5.1 Summary - part 1

The first aim in this thesis was to find out which concentration of anti-CD3/CD28 antibodies should be used for plate bound cell activation, activation method (antibodies coated plate or antibodies covered beads) is more suitable to use for $CD8^+$ T cells and the variance among these two activation methods.

- 5 µg/ml plate bound anti-CD3 and 2 µg/ml soluble anti-CD28 antibodies were the concentrations that gave the best $CD8^+$ T cells stimulation in percentage.
- Antibody coated plate was found to be the most suitable activation method to use for $CD8^+$ T cells. Unfortunately, we could not test the variance among these two methods as two different parameters (flow cytometry and qPCR) were used to verify the cell activation.

5.2 Summary - part 2

Furthermore, the study investigated at which time point the VDR expression was highly induced prior to vitamin D addition and to further investigate the cell response to vitamin D. The genes that are known to be regulated by vitamin D; *TAGAP* and *CYP24A1* were measured in $CD8^+$ T cells.

- VDR expression was highly induced after 40 hours compared to 17 hours of $CD8^+$ T cell stimulation.
- *TAGAP* was downregulated, whereas *CYP24A1* was upregulated with the presence of vitamin D.

5.3 Summary - part 3

The third aim of the thesis was to optimize ChIP protocol to investigate the pulldown of DNA regions that are known to be bound by VDR in $CD4^+$ T can also be candidate DNA regions in $CD8^+$ T cells.

- DNA regions were not pulldown by IP in D1 but all were pulled down in D2. But since we had few donors and one EtOH control to proceed with the ChIP procedure, we cannot fully conclude that these selected DNA regions that were presented in $CD4^+$ T cells, also are candidate DNA region in $CD8^+$ T cells. Therefore, we cannot fully conclude that ChIP procedure is fully optimized and ready to be used. Therefore, more optimization is required.

5.4 Future perspectives

The data in this thesis was exceptionally rich, however, due to time limitations we have only managed to perform full ChIP procedure in one donor (including EtOH and IgG control). Given that our ChIP findings are based on this donor, it should be repeated to confirm whether the procedure are suitable for VDR-ChIP in $CD8^+$ T cells or if is needs further optimizations by including more cells to obtain proper controls, testing different monoclonal and polyclonal antibodies that are specific to VDR and testing primers that can cover other DNA regions in $CD8^+$ T cells than mentioned in this thesis.

The next step is to perform VDR-ChIP sequence in order to investigate whether MS associated genes influencing the binding site of VDR but also to discover and identify new binding site of VDR in $CD8^+$ and $CD4^+$ T cells. It would be interesting to see if $CD8^+$ T cells are mapped to have different VDR binding site than $CD4^+$ T cells and further see how much overlap there is between $CD8^+$ and $CD4^+$ T cells compared to other types of immune cells.

6 Reference list

1. Salou M, Nicol B, Garcia A, Laplaud DA. Involvement of CD8(+) T Cells in Multiple Sclerosis. Front Immunol. 2015;6:604.

2. Laribi B, Sahraian MA, Shekarabi M, Emamnejad R, Marzban M, Sadaghiani S, et al. Characterization of CD4+ and CD8+ T Cell Subsets and Interferon Regulatory Factor 4 (IRF4) in MS Patients Treated with Fingolimod (FTY-720): A Follow-up Study. Iran J Allergy Asthma Immunol. 2018;17(4):346-60.

3. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: the role of infection. Ann Neurol. 2007;61(4):288-99.

4. Fletcher JM, Lalor SJ, Sweeney CM, Tubridy N, Mills KH. T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. Clin Exp Immunol. 2010;162(1):1-11.

5. Hoogs M, Kaur S, Smerbeck A, Weinstock-Guttman B, Benedict RH. Cognition and physical disability in predicting health-related quality of life in multiple sclerosis. International journal of MS care. 2011;13(2):57-63.

6. Popescu BF, Pirko I, Lucchinetti CF. Pathology of multiple sclerosis: where do we stand? Continuum (Minneapolis, Minn). 2013;19(4 Multiple Sclerosis):901-21.

7. Hudgens S, Schuler R, Stokes J, Eremenco S, Hunsche E, Leist TP. Development and Validation of the FSIQ-RMS: A New Patient-Reported Questionnaire to Assess Symptoms and Impacts of Fatigue in Relapsing Multiple Sclerosis. Value Health. 2019;22(4):453-66.

8. Bakshi R, Thompson AJ, Rocca MA, Pelletier D, Dousset V, Barkhof F, et al. MRI in multiple sclerosis: current status and future prospects. The Lancet Neurology. 2008;7(7):615-25.

9. Medana I, Martinic MA, Wekerle H, Neumann H. Transection of major histocompatibility complex class I-induced neurites by cytotoxic T lymphocytes. The American journal of pathology. 2001;159(3):809-15.

10. Domingues RB, Fernandes GBP, Leite F, Tilbery CP, Thomaz RB, Silva GS, et al. The cerebrospinal fluid in multiple sclerosis: far beyond the bands. Einstein (Sao Paulo). 2017;15(1):100-4.

11. Abdelhak A, Huss A, Kassubek J, Tumani H, Otto M. Serum GFAP as a biomarker for disease severity in multiple sclerosis. Scientific reports. 2018;8(1):14798.

12. Lopez-Pedrera C, Perez-Sanchez C, Ramos-Casals M, Santos-Gonzalez M, Rodriguez-Ariza A, Cuadrado MJ. Cardiovascular risk in systemic autoimmune diseases: epigenetic mechanisms of immune regulatory functions. Clin Dev Immunol. 2012;2012:974648.

13. Sethi DK, Gordo S, Schubert DA, Wucherpfennig KW. Crossreactivity of a human autoimmune TCR is dominated by a single TCR loop. Nature Communications. 2013;4(1).

14. Chastain EM, Miller SD. Molecular mimicry as an inducing trigger for CNS autoimmune demyelinating disease. Immunol Rev. 2012;245(1):227-38.

15. Iyer SS, Cheng G. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. Critical reviews in immunology. 2012;32(1):23-63.

16. Buch T, Rieux-Laucat F, Forster I, Rajewsky K. Failure of HY-specific thymocytes to escape negative selection by receptor editing. Immunity. 2002;16(5):707-18.

17. O'Gorman C, Lucas R, Taylor B. Environmental risk factors for multiple sclerosis: a review with a focus on molecular mechanisms. Int J Mol Sci. 2012;13(9):11718-52.

18. Hoglund RA, Maghazachi AA. Multiple sclerosis and the role of immune cells. World J Exp Med. 2014;4(3):27-37.

19. Willer CJ, Dyment DA, Risch NJ, Sadovnick AD, Ebers GC. Twin concordance and sibling recurrence rates in multiple sclerosis. Proc Natl Acad Sci U S A. 2003;100(22):12877-82.

20. International Multiple Sclerosis Genetics C, Wellcome Trust Case Control C, Sawcer S, Hellenthal G, Pirinen M, Spencer CC, et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature. 2011;476(7359):214-9.

21. International Multiple Sclerosis Genetics C, Beecham AH, Patsopoulos NA, Xifara DK, Davis MF, Kemppinen A, et al. Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis. Nat Genet. 2013;45(11):1353-60.

22. Hoppenbrouwers IA, Hintzen RQ. Genetics of multiple sclerosis. Biochim Biophys Acta. 2011;1812(2):194-201.

23. Ascherio A, Munger KL. Epidemiology of Multiple Sclerosis: From Risk Factors to Prevention-An Update. Semin Neurol. 2016;36(2):103-14.

24. Angelini DF, Serafini B, Piras E, Severa M, Coccia EM, Rosicarelli B, et al. Increased CD8+ T cell response to Epstein-Barr virus lytic antigens in the active phase of multiple sclerosis. PLoS Pathog. 2013;9(4):e1003220.

25. Kampman MT, Wilsgaard T, Mellgren SI. Outdoor activities and diet in childhood and adolescence relate to MS risk above the Arctic Circle. J Neurol. 2007;254(4):471-7.

26. Tao C, Simpson S, Jr., van der Mei I, Blizzard L, Havrdova E, Horakova D, et al. Higher latitude is significantly associated with an earlier age of disease onset in multiple sclerosis. J Neurol Neurosurg Psychiatry. 2016;87(12):1343-9.

27. Al Wutayd O, Mohamed AG, Saeedi J, Al Otaibi H, Al Jumah M. Environmental exposures and the risk of multiple sclerosis in Saudi Arabia. BMC Neurol. 2018;18(1):86.

28. Sintzel MB, Rametta M, Reder AT. Vitamin D and Multiple Sclerosis: A Comprehensive Review. Neurol Ther. 2018;7(1):59-85.

29. Kongsbak M, Levring TB, Geisler C, von Essen MR. The Vitamin D Receptor and T Cell Function. Frontiers in Immunology. 2013;4.

30. Karlic H, Varga F. Impact of vitamin D metabolism on clinical epigenetics. Clin Epigenetics. 2011;2(1):55-61.

31. Rolf L, Smolders J, van den Ouweland J, Hupperts R, Damoiseaux J. Correlation of different cellular assays to analyze T cell-related cytokine profiles in vitamin D3-supplemented patients with multiple sclerosis. Mol Immunol. 2019;105:198-204.

32. Dobson R, Giovannoni G, Ramagopalan S. The month of birth effect in multiple sclerosis: systematic review, meta-analysis and effect of latitude. J Neurol Neurosurg Psychiatry. 2013;84(4):427-32.

33. Munger KL, Levin LI, Hollis BW, Howard NS, Ascherio A. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. Jama. 2006;296(23):2832-8.

34. Wingerchuk DM, Lesaux J, Rice GP, Kremenchutzky M, Ebers GC. A pilot study of oral calcitriol (1,25-dihydroxyvitamin D3) for relapsing-remitting multiple sclerosis. J Neurol Neurosurg Psychiatry. 2005;76(9):1294-6.

35. Matías-Guíu J, Oreja-Guevara C, Matias-Guiu JA, Gomez-Pinedo U. Vitamin D and remyelination in multiple sclerosis. Neurología (English Edition). 2018;33(3):177-86.

36. Pierrot-Deseilligny C. Clinical implications of a possible role of vitamin D in multiple sclerosis. J Neurol. 2009;256(9):1468-79.

37. Saccone D, Asani F, Bornman L. Regulation of the vitamin D receptor gene by environment, genetics and epigenetics. Gene. 2015;561(2):171-80.

38. Wucherpfennig KW, Gagnon E, Call MJ, Huseby ES, Call ME. Structural biology of the T-cell receptor: insights into receptor assembly, ligand recognition, and initiation of signaling. Cold Spring Harb Perspect Biol. 2010;2(4):a005140.

39. Mars LT, Saikali P, Liblau RS, Arbour N. Contribution of CD8 T lymphocytes to the immuno-pathogenesis of multiple sclerosis and its animal models. Biochim Biophys Acta. 2011;1812(2):151-61.

40. Xing Y, Hogquist KA. T-cell tolerance: central and peripheral. Cold Spring Harb Perspect Biol. 2012;4(6).

41. Huseby ES, Liggitt D, Brabb T, Schnabel B, Öhlén C, Goverman J. A Pathogenic Role for Myelin-Specific Cd8+T Cells in a Model for Multiple Sclerosis. The Journal of Experimental Medicine. 2001;194(5):669-76.

42. Skulina C, Schmidt S, Dornmair K, Babbe H, Roers A, Rajewsky K, et al. Multiple sclerosis: brain-infiltrating CD8+ T cells persist as clonal expansions in the cerebrospinal fluid and blood. Proc Natl Acad Sci U S A. 2004;101(8):2428-33.

43. Khaibullin T, Ivanova V, Martynova E, Cherepnev G, Khabirov F, Granatov E, et al. Elevated Levels of Proinflammatory Cytokines in Cerebrospinal Fluid of Multiple Sclerosis Patients. Front Immunol. 2017;8:531.

44. Babbe H, Roers A, Waisman A, Lassmann H, Goebels N, Hohlfeld R, et al. Clonal Expansions of Cd8+T Cells Dominate the T Cell Infiltrate in Active Multiple Sclerosis Lesions as Shown by Micromanipulation and Single Cell Polymerase Chain Reaction. The Journal of Experimental Medicine. 2000;192(3):393-404.

45. Denic A, Wootla B, Rodriguez M. CD8(+) T cells in multiple sclerosis. Expert Opin Ther Targets. 2013;17(9):1053-66.

46. Sinha S, Boyden AW, Itani FR, Crawford MP, Karandikar NJ. CD8(+) T-Cells as Immune Regulators of Multiple Sclerosis. Front Immunol. 2015;6:619.

47. Yu Y, Ma X, Gong R, Zhu J, Wei L, Yao J. Recent advances in CD8(+) regulatory T cell research. Oncol Lett. 2018;15(6):8187-94.

48. Chen ML, Yan BS, Kozoriz D, Weiner HL. Novel CD8+ Treg suppress EAE by TGF-beta- and IFN-gamma-dependent mechanisms. Eur J Immunol. 2009;39(12):3423-35.

49. Huseby ES, Huseby PG, Shah S, Smith R, Stadinski BD. Pathogenic CD8 T cells in multiple sclerosis and its experimental models. Front Immunol. 2012;3:64.

50. Kusmartsev SA, Li Y, Chen SH. Gr-1+ Myeloid Cells Derived from Tumor-Bearing Mice Inhibit Primary T Cell Activation Induced Through CD3/CD28 Costimulation. The Journal of Immunology. 2000;165(2):779-85.

51. Fooksman DR, Vardhana S, Vasiliver-Shamis G, Liese J, Blair DA, Waite J, et al. Functional Anatomy of T Cell Activation and Synapse Formation. Annual Review of Immunology. 2010;28(1):79-105.

52. Berge T. The multiple sclerosis susceptibility genes TAGAP and IL2RA are regulated by vitamin D in CD4+ T cells. Science. 2016:118–27.

53. Bretscher PA. A two-step, two-signal model for the primary activation of precursor helper T cells. Proc Natl Acad Sci U S A. 1999;96(1):185-90.

54. Renner C, Jung W, Sahin U, van Lier R, Pfreundschuh M. The role of lymphocyte subsets and adhesion molecules in T cell-dependent cytotoxicity mediated by CD3 and CD28 bispecific monoclonal antibodies. Eur J Immunol. 1995;25(7):2027-33.

55. Chen J, Bruce D, Cantorna MT. Vitamin D receptor expression controls proliferation of naive CD8+ T cells and development of CD8 mediated gastrointestinal inflammation. BMC Immunol. 2014;15:6.

56. Alharbi FM. Update in vitamin D and multiple sclerosis. Neurosciences (Riyadh). 2015;20(4):329-35.

57. Hausler D, Weber MS. Vitamin D Supplementation in Central Nervous System Demyelinating Disease-Enough Is Enough. Int J Mol Sci. 2019;20(1).

58. Prosser DE, Jones G. Enzymes involved in the activation and inactivation of vitamin D. Trends Biochem Sci. 2004;29(12):664-73.

59. Jones G, Prosser DE, Kaufmann M. 25-Hydroxyvitamin D-24-hydroxylase (CYP24A1): its important role in the degradation of vitamin D. Arch Biochem Biophys. 2012;523(1):9-18.

60. Jones G. Metabolism and biomarkers of vitamin D. Scand J Clin Lab Invest Suppl. 2012;243:7-13.

61. Hu XD, Jiang SL, Liu CH, Hu YY, Liu C, Sun MY, et al. Preventive effects of 1,25-(OH)2VD3 against ConA-induced mouse hepatitis through promoting vitamin D receptor gene expression. Acta Pharmacol Sin. 2010;31(6):703-8.

62. Cantorna MT. Why do T cells express the vitamin D receptor? Ann N Y Acad Sci. 2011;1217:77-82.

63. Adorini L. Immunomodulatory effects of vitamin D receptor ligands in autoimmune diseases. International immunopharmacology. 2002;2(7):1017-28.

64. Lambert SA, Jolma A, Campitelli LF, Das PK, Yin Y, Albu M, et al. The Human Transcription Factors. Cell. 2018;172(4):650-65.

65. Haussler MR, Jurutka PW, Mizwicki M, Norman AW. Vitamin D receptor (VDR)-mediated actions of 1alpha,25(OH)(2)vitamin D(3): genomic and non-genomic mechanisms. Best Pract Res Clin Endocrinol Metab. 2011;25(4):543-59.

66. Carlberg C, Campbell MJ. Vitamin D receptor signaling mechanisms: integrated actions of a well-defined transcription factor. Steroids. 2013;78(2):127-36.

67. Lu M, McComish BJ, Burdon KP, Taylor BV, Korner H. The Association Between Vitamin D and Multiple Sclerosis Risk: 1,25(OH)2D3 Induces Super-Enhancers Bound by VDR. Front Immunol. 2019;10:488.

68. Hwang YG, Hsu HC, Lim FC, Wu Q, Yang P, Fisher G, et al. Increased vitamin D is associated with decline of naive, but accumulation of effector, CD8 T cells during early aging. Adv Aging Res. 2013;2(2):72-80.

69. Handel AE, Sandve GK, Disanto G, Berlanga-Taylor AJ, Gallone G, Hanwell H, et al. Vitamin D receptor ChIP-seq in primary CD4+ cells: relationship to serum 25-hydroxyvitamin D levels and autoimmune disease. BMC Medicine. 2013;11:163.

70. Tavera-Mendoza LE, Mader S, White JH. Genome-wide approaches for identification of nuclear receptor target genes. Nucl Recept Signal. 2006;4:e018.

71. Nelson JD, Denisenko O, Bomsztyk K. Protocol for the fast chromatin immunoprecipitation (ChIP) method. Nat Protoc. 2006;1(1):179-85.

72. Hoffman EA, Frey BL, Smith LM, Auble DT. Formaldehyde crosslinking: a tool for the study of chromatin complexes. J Biol Chem. 2015;290(44):26404-11.

73. Forster M, Boora RK, Petrov JC, Fodil N, Albanese I, Kim J, et al. A role for the histone H2A deubiquitinase MYSM1 in maintenance of CD8(+) T cells. Immunology. 2017;151(1):110-21.

74. Pchelintsev NA, Adams PD, Nelson DM. Critical Parameters for Efficient Sonication and Improved Chromatin Immunoprecipitation of High Molecular Weight Proteins. PLoS One. 2016;11(1).

75. Zhu J, Huang X, Yang Y. NKG2D is Required for NK Cell Activation and Function in Response to E1-deleted Adenovirus. J Immunol. 2010;185(12):7480-6.

76. Aksoy BA, Aksoy P, Wyatt M, Paulos C, Hammerbacher J. Human Primary T cells: A practical Guide. 2018.

77. Klockenbusch C, Kast J. Optimization of formaldehyde cross-linking for protein interaction analysis of non-tagged integrin beta1. J Biomed Biotechnol. 2010;2010:927585.

7 Appendix

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Appendix 1. Materials, machines and computer software

Materials	Products	Cat. no
	Cell culture	
CD8 Isolation Kit	Stem Cell™ Technologies	17853
Dynabeads™ Protein A	Thermo Fisher Scientific	00564257
T-activator CD3/CD38 Dynabeads	Thermo Fisher Scientific	11456D
RPMI medium 1640 (1x)	Gibco® Life technologies™	21875-034
X-VIVO ™	Lonza	BE02-060F
Dulbecco's phosphate buffered saline (PBS)	Sigma Life Science	D8537-500ML
Lymphoprep	Stem Cell™ Technologies	12IMS05
Counting slides, dual chamber for cell counter	Bio-Rad	145-0015
Tryphan Blue Solution (0.4%)	Sigma-Aldrich	T8154
1α,25-Dhydroxyvitamin D_3	Sigma	D1530-10UG
	Buffers and Standards	
Foetal bovine serum (FBS)	Gibco ® Thermo Scientific	SV30160.03
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich	D8537-500ML
	RNA isolation	
Qiashredder	Qiagen	79656
RNAprotect® Cell Reagent 250ml	Qiagen	74034
RNeasy Plus mini kit	Qiagen	74134
RNase AWAY®	VWR	7002
	Gene expression	
TaqMan® Gene expression Master Mix	Applied biosystems	4369016
10x PCR Rxn Buffer	Invitrogen	Y02028
Platinum Taq	Invitrogen	10966-034
DNase, Rnase and protein free water	5 PRIME	2500010

SDS-PAGE and Western blot

Immuno-blot® PVDF membrane	Bio-Rad	1704157
Precision Plus Protein™ Dual Colour	Bio-Rad	161-0374
Restore™ PLUS Western Blot Stripping	Thermo Scientific	46430
Anti-actin produced in rabbit	Sigma	A2066
Skim milk powder	Sigma Aldrich	70166-500G
Clarity™Western ECL Substrate	Bio-Rad	102030889
Tris buffered saline with Tween® 20 (TBS-T)	Medicago	09-7510-100
TBS-Tween™ Tablets	Medicago	09-7510-100
Ponceau staining solution	Sigma-Aldrich	P7170-1L

ChIP

Formaldehyde	Sigma Life Science	F8775-25ML
Glycine	Sigma-Aldrich	G7126-1KG
Anti-VDR	Santa Cruz Biotechnology	sc-13133
Anti-IgG	Santa Cruz Biotechnology	sc-2025

DNA purification

Phenol Chloroform	Sigma Life Science	P3803-100ML
Chloroform	Sigma Life Science	C0549-1PT
B-mercaptoethanol	Sigma Aldrich	M3148-100ML
Sodium acetate	BioXtra	SLBQ8253V
Glycogen	Invitrogen	10814-010
MicroChIP Pure column	Diagenode	C03040001

Agarose gel electrophoresis

Magnesium Chloride ($MgCl_2$)	Invitrogen	Y02016
Ethidium bromide (EtBr)	MP- Biomedical, LLC	190202
Agarose, Seakem® LE	Lonza	50004

GeneRuler low range	Thermo Scientific	SM1193
GeneRuler 100pb DNA ladder	Thermo Scientific	SM0241
6x DNA loading dye	Thermo Scientific	R0611

Chemicals

Complete, EDTA free Tablets	Roche	11873580001
PMSF	Sigma Aldrich	P7626-5G

Machines	**Producer**
ChemiDoc™ Touch Imaging System	Bio-Rad
NanoDrop 2000c	Thermo Scientific
2720 Thermal Cycler	Applied Biosystem
Attune® Acoutic Focusing Cytometer	Applied Biosystem
Minichiller 300	Diagenode
Ultrasonic Processor (UP)100H	Hielscher Ultrasound Technology
TC20™Automated Cell Counter	Bio-Rad
Software	**company**
Image Lab 5.2.1	Bio-Rad Laboratories
NanoDrop 2000c	Thermo Scientific
Viia™ 7 Software	Applied Biosystem

Appendix 2: Recipes

RIPA BUFFER	**Stock**	**100 ml**	**1 ml**	**4ºC**
0.1% SDS	10%	1 ml		
0.1% sodium deoxycholate		0.1 g		
1% Triton X-100	100%	1 ml		
1 mM EDTA (pH 8)	0.5 M	200 µl		
0.5 mM EGTA (pH 8)	0.5 M	100 µl		
140 mM NaCl	5 M	2.8 ml		
10 mM Tris-HCl (pH 8)	1 M	1 ml		
dH_2O		93.9 ml		
Protease inhibitor cocktail	*25x*		*40 µl*	*Add to 1 ml of RIPA*
1 mM PMSF	*100 mM*		*10 µl*	*buffer just before use*

ELUTION BUFFER	**Stock**	**50 ml**	**4ºC**
50 mM NaCl	5 M	500 µl	
20 mM Tris-HCl (pH 7.5)	1 M	1 ml	
5 mM EDTA (pH 8)	0.5 M	500 µl	
dH_2O		42 ml	
1% SDS	*10%*	*5 ml*	*Add just before use*

1x Tris/Glycine/SDS running buffer

(25 mM Tris, 192 mM 0.1% SDS)	**1 liter**
10x Tris/Glycine/SDS running buffer	100 ml
dH_2O	990 ml

3% skimmed milk in 1 x TBS-T

(10 mM Tris, 150 mM NaCl, Tween 20)	**500 ml**
Skimmed milk powder	15 g
1x TBS-T	up to 500 ml

Appendix 3. Standard curves of *PGK1*, *VDR*, *TAGAP* and *CYP24A1* from three donors after vitamin D treatment
Donor 1

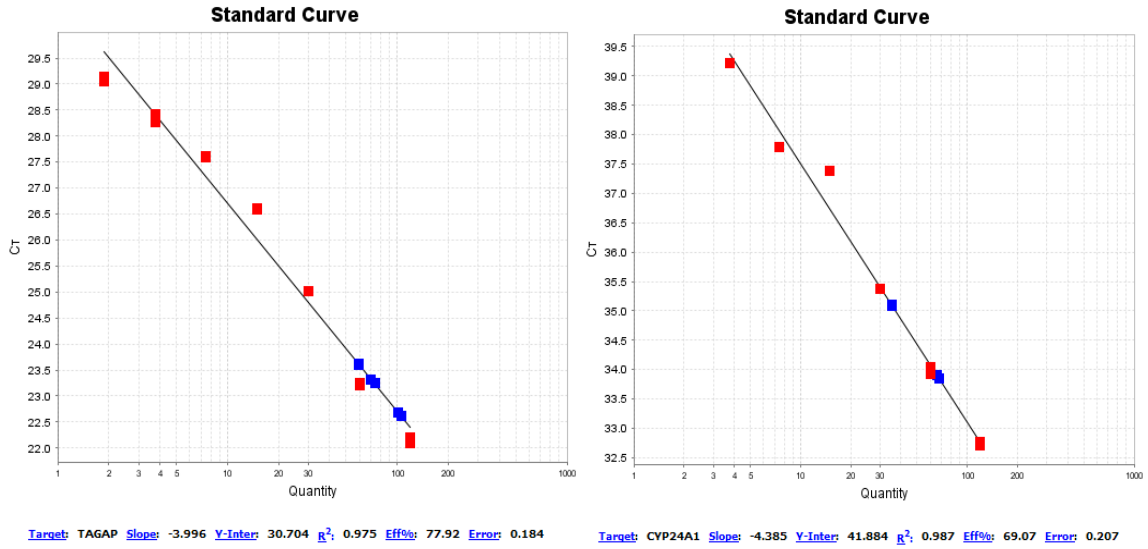

Figure 5.1: The standard curve of TAGAP and CYP24A1 from donor 1. *Red dots are standard curve samples, whereas the blue dots present the samples.*

Donor 2

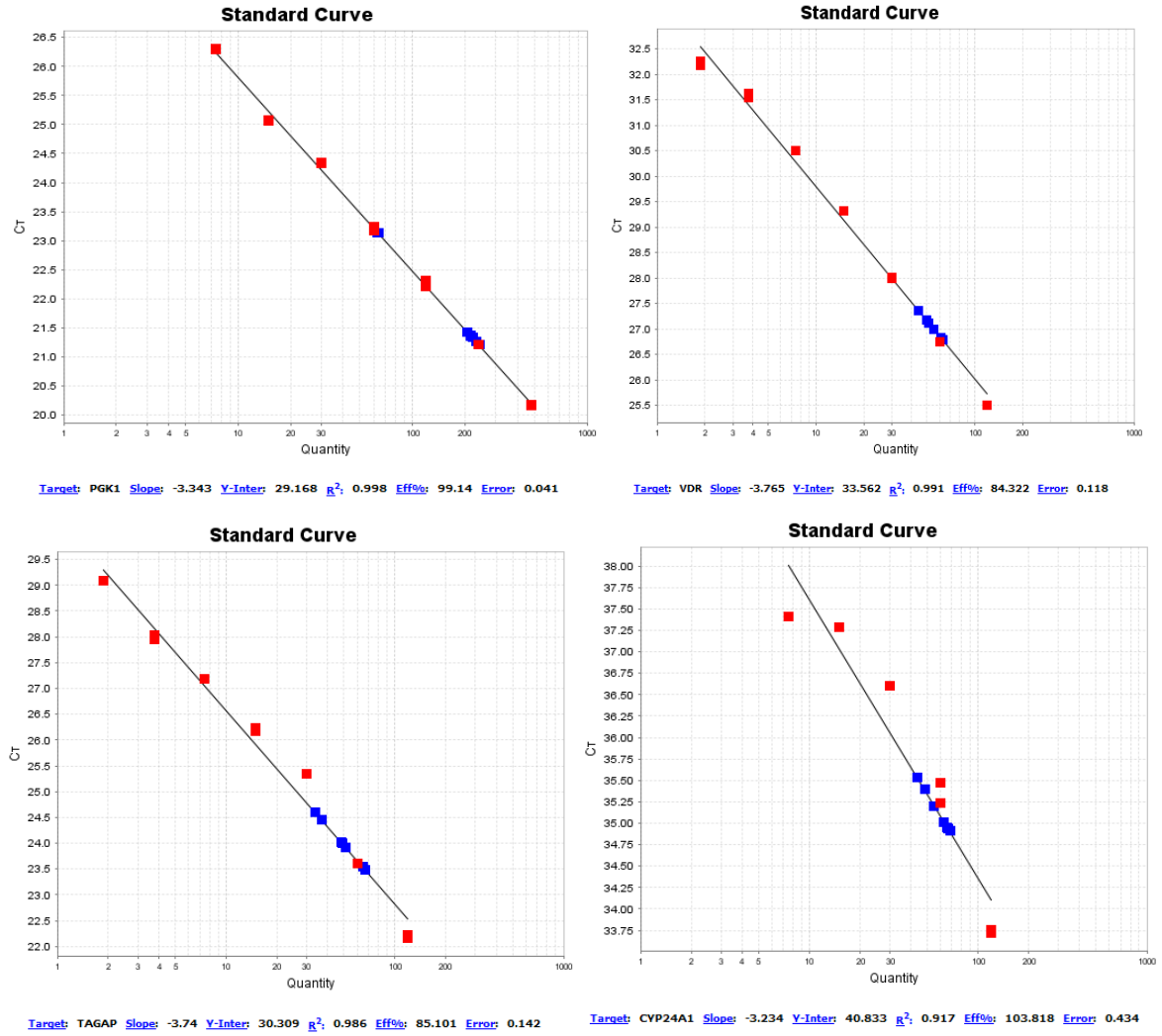

Figure 5.2: Standard curve of PGK1, VDR, TAGAP and CYP24A1 from donor 2. *Red dots are standard curve samples, whereas the blue dots present the samples.*

Donor 3

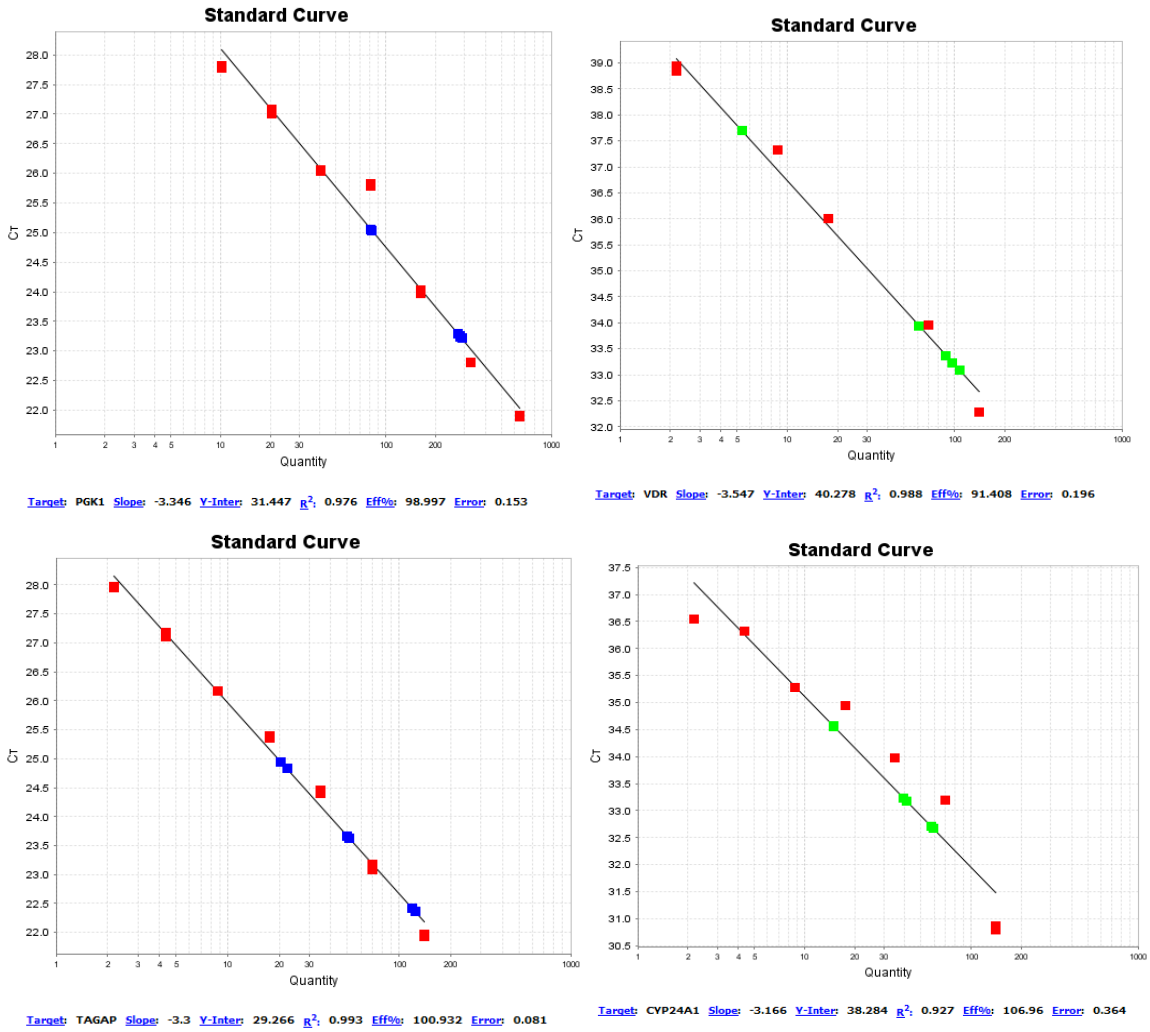

Figure 5.3: Standard curve of PGK1, VDR, TAGAP and CYP24A1 from donor 3. *Red dots are standard curve samples, whereas the blue dots and green dots present the samples.*

Appendix 4. Statistics

All statistical calculations are done in Excel

Table 5.1: Paired t-test performed on VDR expression before (0 hours) and after (40 hours) anti-CD3/CD28 antibodies stimulation. *H0= $\mu_{diff} = 0$. The p-value is much lower than 0.05, hence the expression of VDR before is significantly lower than VDR expression after stimulation.*

	Non-stimulated (0 hours)	***Stimulated (40 hours)***
Mean	1	6,930505532
Variance	0	1,956213624
Observations	4	4
Hypothesizes difference between means	0	
Degrees of freedom	3	
t-Stat	-8,48034599	
P(T<=t) two sided	**0,003442757**	
T-critical, two sided	3,182446305	

Table 5.2 Paired t-test performed on VDR expression before (0 hours) and after (17 hours) anti-CD3/CD28 antibodies stimulation. *H0= $\mu_{diff} = 0$. The p-value is a bit lower than 0.05, hence the expression of VDR before is significantly lower than VDR express after stimulation.*

	Non-stimulated (0 hours)	***Stimulated (17 hours)***
Mean	1	4,918467705
Variance	0	5,703996724
Observasions	4	4
Hypothesizes difference between means	0	
Degrees of freedom	3	
t-Stat	-3,281380808	
P(T<=t) two sided	**0,04637762**	
T-kritisk, two sided	3,182446305	

Table 5.3 Paired t-test performed on VDR expression after (17 and 40 hours) anti-CD3/CD28 antibodies stimulation. *H0= $\mu_{diff} = 0$. The p-value is much higher than 0.05, suggesting that there is no significant difference of VDR expression after 17 and 40 hours of stimulation.*

	Stimulated (17 hours)	***Stimulated (40 hours)***
Mean	4,918467705	6,930505532
Variance	5,703996724	1,956213624
Observasions	4	4
Hypothesizes difference between means	0	
Degrees of freedom	5	
t-Stat	-1,453937672	
P(T<=t) two sided	**0,205707744**	
T-kritisk, two sided	2,570581836	

Table 5.4: Paired t-test performed on TAGAP expression before and after vitamin D treatment*. H0= $\mu_{diff} = 0$. The p-value is lower than 0.05, hence the presence of TAGAP before vitamin D is significantly higher than after vitamin D treatment.*

	Before vitamin D treatment	***After vitamin D treatment***
Mean	1	0,53338179
Variance	0	0,016808368
Observations	3	3
Hypothesizes difference between means	0	
Degrees of freedom	2	
t-Stat	6,233895731	
P(T<=t) two sided	**0,024779964**	
T-critical, two sided	4,30265273	

Table 5.5: The cell viability after activating using plate coated antibodies and Dynabead covered antibodies. *H0= μ_{diff} = 0. The p-value is higher than 0.05, suggesting that there is no significant difference of cell viability among plate and Dynabead activated cells.*

	Plate bound activation	***Dynabead bound activation***
Mean	81,7	87
Variance	9,33	7
Observations	3	3
Hypothesizes difference between means	0	
Degrees of freedom	4	
t-Stat	-2,285714286	
P(T<=t) two sided	**0,084254022**	
T-critical, two sided	2,776445105	

Appendix 5. Result

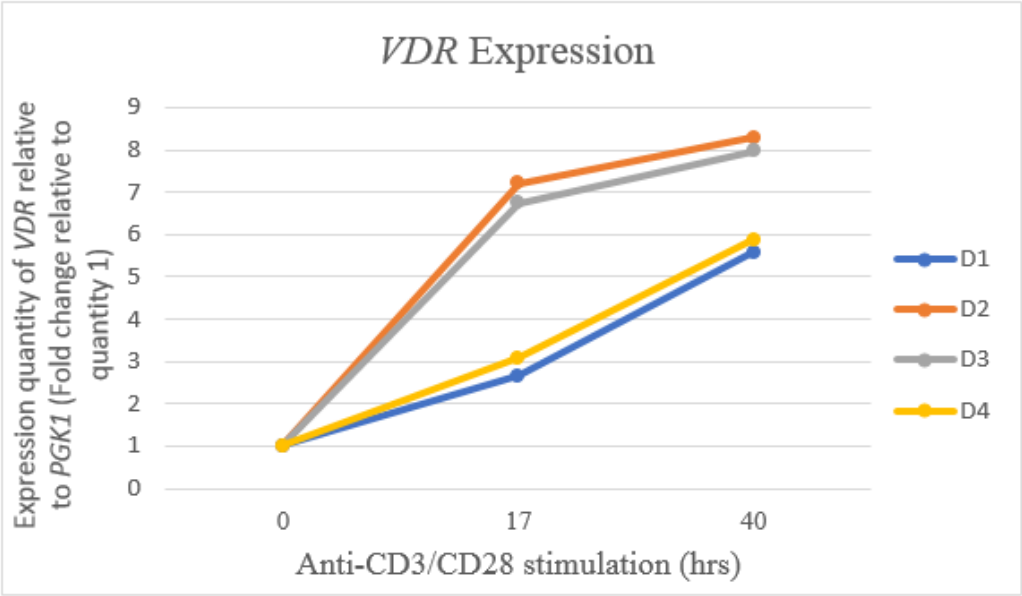

Figure 5.4: VDR expression is highest after 40 hours of stimulation. *The line graph showing fold change in VDR gene expression from four donors at the indicated time points. Values represents fold change relative to nonactivated cells (time point 0).*