Reverse Transcriptase Activity Assays for Retrovirus Quantitation and Characterization

BY
ANDERS MALMSTEN
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Abstract

Reverse transcriptase (RT) is a crucial enzyme for retrovirus replication, and its presence in the virion is indispensable for infectivity. This thesis illustrates the use of RT activity assays as tools for quantitation and characterization of different retroviruses, particularly HIV.

A non radioactive assay, using microtiter plates, for the RT of Moloney murine leukemia virus (MMuLV) was developed. Assay conditions for MMuLV and HIV-1 RT, together with isozyme specific RT activity blocking antibodies, were shown useful for discrimination between RTs from different retrovirus genera. RT activity assay for HIV-1 was found to quantitate different subtypes more equally efficient than p24 antigen assays did.

Viral load (VL), the amount of HIV particles in the blood, is an important marker of the clinical status of an infected person. A method for VL determination based on RT activity (ExaVir Load) was developed. After plasma pretreatment, to inactivate cellular DNA polymerases, virions in patient plasma were immobilized on a gel, which was washed to remove disturbing factors. The virions were lysed with a detergent containing buffer and the lysate eluted. Finally, the RT activity in the lysate was determined and found to correlate strongly to VL by RNA according to a PCR based standard method (Roche Amplicor 1.5). The second version of the method was able to measure VL down to approximately 400 HIV-1 RNA copies/ml. The usefulness of RT from the VL procedure for determination of susceptibility towards anti-HIV drugs was demonstrated, and the results were in agreement with genotypic data.

Due to its technical simplicity, and ability to detect a broad range of HIV-1 subtypes, ExaVir Load and the drug susceptibility application are interesting for clinical use, particularly but not only in resource limited settings. The concept is also potentially useful for research purposes, e.g. in combination with specific RT assay conditions.

Keywords: retrovirus, reverse transcriptase, enzyme activity assay, MuLV, HIV, RT purification, viral load, drug susceptibility

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Det är mycket med replikationsenzymologien
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# CONTENTS

INTRODUCTION.................................................................................................................. 11
  About this thesis........................................................................................................... 11
  History of exogenous retroviruses........................................................................... 11
    Non-HIV .................................................................................................................. 11
    HIV ......................................................................................................................... 12
  Common features of retroviruses.............................................................................. 14
    Genome.................................................................................................................. 14
    Structure................................................................................................................. 15
    Replication cycle .................................................................................................. 15
  Retrovirus overview ................................................................................................. 16
    Endogenous ............................................................................................................ 16
    Exogenous .............................................................................................................. 17
  γ–retroviruses ............................................................................................................ 17
  Lentiviruses ............................................................................................................... 19
    Overview ............................................................................................................... 19
    HIV and its infection ............................................................................................. 20
      Course of HIV disease ......................................................................................... 20
      HIV-1 subtypes .................................................................................................... 21
      HIV-2 .................................................................................................................. 21
  RT enzymes and assays ............................................................................................ 22
    Enzyme .................................................................................................................. 22
      Function .............................................................................................................. 22
      Structure ............................................................................................................. 23
    RT activity assays .................................................................................................. 23
      Radioactive ......................................................................................................... 24
      Non-radioactive ................................................................................................. 25
      Methods involving PCR ...................................................................................... 28
      RT activity blocking antibodies .......................................................................... 30
  Diagnosis and monitoring of HIV infection ............................................................. 30
    Diagnosis ............................................................................................................... 30
    CD4 cell count ...................................................................................................... 31
    Viral load ............................................................................................................... 31
      RNA based ......................................................................................................... 31
      Antigen based .................................................................................................... 33
      RT based ............................................................................................................. 33
      Principles compared .......................................................................................... 34
  HIV therapy and drug resistance .............................................................................. 35
    Anti-HIV drugs ...................................................................................................... 35
    RT directed ............................................................................................................ 35
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>Ac</td>
<td>acetate</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AMV</td>
<td>avian myeloblastosis virus</td>
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<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AZT</td>
<td>azidodeoxythymidine</td>
</tr>
<tr>
<td>bDNA</td>
<td>branched DNA</td>
</tr>
<tr>
<td>bio</td>
<td>biotin</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BrdUTP</td>
<td>bromodeoxyuridine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>D</td>
<td>Dalton</td>
</tr>
<tr>
<td>d4T</td>
<td>2',3'-didehydro-3'-deoxythymidine</td>
</tr>
<tr>
<td>dig</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>DLV</td>
<td>delavirdine</td>
</tr>
<tr>
<td>DS</td>
<td>drug susceptibility (or sensitivity)</td>
</tr>
<tr>
<td>DTE</td>
<td>1,4 -dithioerythitol</td>
</tr>
<tr>
<td>DTNB</td>
<td>dithiobisnitrobenzoic acid</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine triphosphate</td>
</tr>
<tr>
<td>EFV</td>
<td>efavirenz</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>Env</td>
<td>envelope</td>
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<tr>
<td>EVL1 (2)</td>
<td>ExaVir Load version 1 (2)</td>
</tr>
<tr>
<td>FDA</td>
<td>food and drug administration (USA)</td>
</tr>
<tr>
<td>FeLV</td>
<td>feline leukemia virus</td>
</tr>
<tr>
<td>Fig.</td>
<td>figure</td>
</tr>
<tr>
<td>FIV</td>
<td>feline immunodeficiency virus</td>
</tr>
<tr>
<td>Gag</td>
<td>group antigen</td>
</tr>
<tr>
<td>(H)ERV</td>
<td>(human) endogenous retrovirus</td>
</tr>
<tr>
<td>HIV-1 (2)</td>
<td>human immunodeficiency virus type 1 (2)</td>
</tr>
<tr>
<td>HS</td>
<td>high sensitivity</td>
</tr>
<tr>
<td>HTLV</td>
<td>human T-lymphotropic (or leukemia) virus</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>IC50</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IN</td>
<td>integrase</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>MAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>(M)MuLV</td>
<td>(Moloney) murine leukemia virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NASBA</td>
<td>nucleic acid sequence based amplification</td>
</tr>
<tr>
<td>(N)NRTI</td>
<td>(non) nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NVP</td>
<td>nevirapine</td>
</tr>
<tr>
<td>odG</td>
<td>oligodeoxyguanylic acid</td>
</tr>
<tr>
<td>odT</td>
<td>oligodeoxythymidylic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PERT</td>
<td>product enhanced reverse transcriptase</td>
</tr>
<tr>
<td>PERV</td>
<td>porcine endogenous retrovirus</td>
</tr>
<tr>
<td>PI</td>
<td>protease inhibitor</td>
</tr>
<tr>
<td>pNPP</td>
<td>para nitrophenyl phosphate</td>
</tr>
<tr>
<td>Pol</td>
<td>polymerase</td>
</tr>
<tr>
<td>prA</td>
<td>polyriboadenylic acid</td>
</tr>
<tr>
<td>prC</td>
<td>polyribocytidylic acid</td>
</tr>
<tr>
<td>PR</td>
<td>protease</td>
</tr>
<tr>
<td>RNase H</td>
<td>ribonuclease H</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase (RNA dependent DNA polymerase)</td>
</tr>
<tr>
<td>RTb-Ab</td>
<td>RT activity blocking antibody</td>
</tr>
<tr>
<td>SIV</td>
<td>simian immunodeficiency virus</td>
</tr>
<tr>
<td>strav</td>
<td>streptavidine</td>
</tr>
<tr>
<td>TP</td>
<td>triphosphate</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
</tbody>
</table>
INTRODUCTION

About this thesis

The thesis you are reading is mainly a description of the development and application of methods for quantitation and characterization of retroviruses, based on enzymatic assays for reverse transcriptase activity. Particularly, the current work shows that reverse transcriptase activity is a practically useful marker for HIV in plasma, giving alternatives to the established methods for viral load and drug susceptibility determinations. These methods are intended for clinical use in resource limited settings in developing countries, but could have advantages, making them interesting also for use in the developed world.

The thesis is method oriented, which implies that most of the work presented consists of the development of methods, and comparisons of these to state of the art. As such, this does not include any groundbreaking basic scientific data, but the methods described may be valuable as tools for research and, hopefully, as small a contribution to the worldwide fight against AIDS.

History of exogenous retroviruses

Non-HIV

Some of the earliest discovered viruses were in fact retroviruses. In the year 1908, the Danish veterinarians Bang and Ellermann found that a certain form of leukemia in chicken was caused by a transmissible agent [16]. This agent was avian leukosis virus (ALV). In 1909, Rous was able to transfer sarcomas between chickens by injection of a cell-free filtrate from an affected hen to a healthy [219]. The filtrate was later shown to contain the retrovirus, Rous sarcoma virus (RSV). This virus contains an oncogene that has the ability to disturb the cell cycle in such a way that the infected cell is immortalized [251]. Both these viruses belong to the genus of α-retroviruses, previously called avian C-type retroviruses (Table 2).

Later mice became major study objects in retrovirology. In 1960, Moloney published the isolation of a strain of murine leukemia virus that carries his name [165]. This virus, which normally causes T-cell lymphoma, has been very important in science since then, and its RT is one of the standard enzymes used in many kinds of studies. Several different strains of MuLV have been isolated, and it is probably the best studied of all retroviruses (perhaps now with the exception of HIV). It belongs to the genus of γ-retroviruses, previously called mammalian C-type retroviruses.
Baltimore and Temin described the function of retroviruses in 1970 [14, 270]. The word “retroviruses” was introduced to indicate the fact that they display a “retro”, backwards, flow of genetic information. Retroviruses were shown to break one of the basic “laws” of biology, namely that DNA acts as template for RNA. One typical feature of retroviruses is that their genome consists of RNA, that is reverse transcribed into DNA and integrated into the genome of the host cell. The first part of this process is performed by the enzyme reverse transcriptase (RT) which is the main focus of this thesis.

For a long time, science did not know about any human retrovirus. However, in 1980 the human T-cell lymphotropic virus type 1 (HTLV-I) was described [199], and soon also HTLV-II [118]. These viruses were similar to a previously known virus in cattle, bovine leukemia virus (BLV) [283].

From cattle was also earlier isolated bovine immunodeficiency virus (BIV), belonging to a group called lentiviruses [30, 283]. Lentiviruses had been found in some domestic animals, including horses (equine infectious anemia virus, EIAV), sheep (Visna/maedi virus) and goats (caprine arthritis encephalitis virus, CAEV) [44, 248, 281]. EIAV was discovered very early, at about the same time as ALV and RSV. The word “lenti” means “slow” in Latin. The name is derived from the fact that most lentiviruses exhibit a slow disease progression after infection, often with several years before the emergence of a visible disease.

HIV

In 1981, several cases of some very rare infections and malignancies were noticed among young homosexual men in large city areas of the US. This puzzled the physicians since the disorders in question previously almost only affected elderly persons with deficient immune systems. After some time, a new disorder was defined according to a list of symptoms. The new disorder was called AIDS, acquired immunodeficiency syndrome. There were several theories about the origin of the syndrome, including lifestyle related reasons such as use of recreational drugs. However, in 1983 HIV-1 was isolated by a group in France [18] and at about the same time by a group in the US [72, 200]. The new virus was first called either LAV (lymphadenopathy associated virus) or HTLV-III. It was however soon found that the virus belonged to the previously known genus of lentiviruses. The discovery of HIV-1 [73] was a few years later followed by that of HIV-2 [39]. The nature of the virus and the wide spread of AIDS triggered a massive research campaign, that is very much still going on.

HIV causes AIDS by damaging the immune system and thus making the body susceptible to infections and tumours that otherwise would not harm the person. Although this must be regarded as generally accepted [9], there are some scientists who still do not recognize the link between HIV and AIDS [57, 58], or even deny that HIV has ever been proven to exist [185].
Sadly, the spread of HIV has proved to be even more massive than the efforts put into the struggle against it, and the odds of this struggle are not improved by the presence of destructive forces inside the scientific community. The influence of these forces on some political decision makers makes the situation even worse.

In 2004, the number of HIV infected in the world was calculated by the UN to be 39 million. More than 20 million people have died from AIDS, and about 3 million died in 2004. The vast majority of the infected live in developing countries in Asia and Africa. At the moment, the most rapid spread of HIV is believed to take place in Asia.

However, the situation is particularly gruesome in Africa south of the Sahara. In Botswana, about 35% of the entire population is HIV infected. In South Africa, the number of infected is over 5 million. The situation is similar also in e.g. Malawi, Namibia and Zimbabwe. These terrifying figures tell us that this part of the world is suffering from a disaster. However, the massive research has resulted in the emergence of functional treatment against HIV, treatment that has the ability to slow down disease progression and prolong the life of the patient. The treatments have been accessible in the developed world for some time. Currently large programs for treatment are underway also in many developing countries. This could have important impact not only on the life of the infected person, but also on the society of the affected area, as the possibility of economic growth and the taking care of children is in great jeopardy in areas where AIDS is common. When patients are being treated, the need for cost efficient methods for their monitoring arises, as HIV commonly develops resistance to the therapies. In this context, this thesis could possibly be able to make a contribution.

![Figure 1. The proviral genomes of MMuLV and HIV-1.](image-url)
Common features of retroviruses

Genome

Typical retroviral genomes, exemplified by MMuLV and HIV-1, are seen in Fig. 1. The length of the MMuLV genome is 8.3 kbp, while the genome of HIV-1 is 9.2 kbp. MMuLV is an example of a simple retroviruses, carrying only the genes \textit{gag}, \textit{pro}, \textit{pol} and \textit{env}. The HIV-1 genome is complex, with several additional genes present, whose functions are to regulate details in the virus replication. A description of the genes of MMuLV and HIV-1 is seen in Table 1. For a description in detail of the genes of HIV-1, see [69]. In the case of HIV-1, the \textit{pro} gene is a part of the \textit{pol} gene. All retrovirus genomes have long terminal repeats (LTRs) at their ends. These are structures involved in the transcription of the RNA. Every retrovirus virion carries two copies of genomic RNA inside its core. The exact role of the presence of two RNA molecules is not known, and only one provirus DNA is produced from each virion during the transcription.

Some retroviruses do also, beside the mentioned genes, contain oncogenes. This is e.g. the case for RSV and some strains of MuLV.

Table 1. Genes encoded by HIV-1 and MMuLV

<table>
<thead>
<tr>
<th>Gene</th>
<th>Virus</th>
<th>Functional protein(s) in HIV-1</th>
<th>Function(s)</th>
</tr>
</thead>
</table>
| \textit{env} | HIV-1 / MMuLV | SU (gp120)  
TM (gp41) | Binds to CD4 of host cell  
Involved in fusion with host cell membrane |
| \textit{gag} | HIV-1 / MMuLV | MA (p17)  
CA (p24)  
NC (p7)  
p6 | Matrix protein associated to envelope  
Structural protein of virus capsid  
Forms complex with RNA  
Involved in viral packing of Vpr |
| \textit{pol} | HIV-1 / MMuLV | RT (p66/51)  
IN (p32)  
PR (p11) | Reverse transcription and RNase-H activity  
Integration of provirus in host cell genome  
Proteolytic cleavage of precursor proteins |
| \textit{nef} | HIV-1 | Nef (p27) | Increases infectivity, CD4 degradation and proviral DNA synthesis, packed inside virion |
| \textit{rev} | HIV-1 | Rev (p19) | Regulates processing of viral mRNA |
| \textit{tat} | HIV-1 | Tat (p14) | Transcriptional transactivator and enhancer |
| \textit{vif} | HIV-1 | Vif (p23) | Enhances infectivity, packed inside virion |
| \textit{vpr} | HIV-1 | Vpr (p15) | Mediates transport of viral proteins to nucleus, arrests cell cycle, packed inside virion |
| \textit{vpu} | HIV-1 | Vpu (p16) | Enhances CD4 degradation and particle release |
Structure

Retroviruses are spheres with spikes, with a diameter of about 100 nm. The basic structure of a retrovirus, in this case HIV-1, is shown in Fig. 2. The outer envelope consists of trimers of the TM protein, which are attached to SU and anchored in a lipid membrane derived from the host cell. Inside the envelope is a spherical matrix built up by MA. The matrix surrounds the core, which is cone shaped in lentiviruses and consist of the CA protein. Inside it the two RNA molecules are found, which are, in spite of the figure, connected to each other at their 5’ ends. They are attached to many molecules of NC. Also inside the core are the RT, PR and IN proteins, tRNA molecules used as RT primers and, for some retroviruses, additional proteins.

The shape and position of the inner core differ between retrovirus groups (Table 2). Most groups have a morphology called C-type, which means that the core is roughly spherical and centrally located. Other morphologies are B-type (eccentric spherical core) and D-type (bar-shaped core). The spumaviruses have spherical cores similar to the C-type morphology, but with other features (not discussed here) making them a morphological group of their own.

Figure 2. Schematic structure of an HIV-1 virion. (Modified from [42]).

Replication cycle

All exogenous retroviruses have the main events of replication in common. A typical replication cycle, most closely that of HIV-1, will be briefly described in the following. Full review can be found in [85].

The cycle starts with the binding of the virus by its surface glycoprotein (SU) to a receptor (for HIV CD4, for MuLV ecoR) on the surface of a susceptible cell [48, 286]. For most viruses, other receptors are also involved in this process. For HIV, these are mainly CCR5 and CXCR4; at least one is
needed on the target cell for infection [38, 166]. Conformational changes of the protein receptors facilitates the fusion between the virus and cell membranes, and the core is able to enter the cytoplasm.

Once inside, the RNA and RT are set free and reverse transcription occurs. The result is double stranded DNA corresponding to the virus genome. This DNA is transported to the cell nucleus, for HIV with the help of the Vpr protein, and integrated into the genome of the cell by the action of the viral IN. The viral genome, or provirus, is thus a part of the cell genome for life.

The transcription of the provirus is initiated by cellular factors, for HIV together with the viral Tat-protein. Viral mRNA is produced from the provirus by the action of cellular RNA polymerase. In the case of HIV, the resulting mRNA is then spliced, differently depending on the stage of the transcription. In the early part of replication, a lot of mRNA for the accessory proteins is produced. By the influence of Rev, later more full length transcripts for the other proteins are made. The mRNAs are translated in the cytoplasm to precursor proteins, from which the final functional enzymes and structural proteins are cleaved by PR. The RT is before cleavage a part of a polyprotein together with Gag. The Env protein is glycosylated by cellular enzymes, and inserted in the membrane as the new virion is assembled and finally budded from the host cell, ready for the next round of infection.

**Retrovirus overview**

**Endogenous**

All animals have a multitude of retroviruses, and related sequences, inherited in their cellular genomes. This group of sequences is called retroelements and is extremely diverse [17, 82, 280]. Most of these elements are not complete retroviruses, but rather truncated residues of proviruses or related sequences. The existence of retroelements probably reflects the integration of proviruses through the history of evolution. However, sequences remain that are complete endogenous proviruses. Human endogenous retroviruses are occasionally expressed, for instance in placenta [97], in fetal tissue [7] and in certain cell lines [66, 189]. In some cases RT activity has been detected which seems to be of endogenous origin [140, 143, 189]. Functional RT from the HERV-K group [302] has been cloned and recombinantly expressed [23]. The RT was found to contain a functional RNase H domain and have a preference for Mg$^{2+}$ in assay.

Endogenous retroviruses expressed in humans have been connected to a variety of disorders, including autoimmune states like systemic lupus erythematosus, rheumatoid arthritis, and MS [128, 180, 182, 204], cancer in breast and prostate [287, 288], and schizophrenia [121]. The relation between endogenous retroviruses and disease remains generally unclear, and in no case has any causative relationship been definitely established.
Exogenous

Exogenous retroviruses (Table 2) have been found in most species, and cause disease in their host in many, but far from all cases. Sometimes a virus that is benign in its natural host is able to cause disease in a related species, as is the case for SIV and apparently HIV.

Many retroviruses induce various kinds of tumours. Most of these do this in an indirect way, by activating cellular genes after the insertion of the provirus into the host cell genome [33]. This is the case for the only known tumour inducing human retroviruses, HTLV-I and -II.

Of the retroviruses causing tumours, two interesting ones could be mentioned, namely MMTV and JSRV, both belonging to the β-retroviruses. They cause breast tumours in mice [155] and lung tumours in sheep [67, 183], respectively, and it has been suspected that they could have human counterparts involved in corresponding diseases. Although sequences similar to MMTV [141, 301] and proteins immunologically related to JSRV [50] have been reported, the evidence for involvement in human disease are so far not convincing [150, 151, 167].

There are several systems for classification of retroviruses. Initially, the viruses were divided in categories depending on morphological features and connection with disease. There is also a classification system based on the identity of the tRNAs used as primers for the RT; this system is mainly used for endogenous retroviruses.

However, in recent years a new classification system has been introduced. It is based on genetic features and is shown in Table 2. The virus family of Retroviridae is here divided into seven genera, of which five are named in Greek alphabetic order. The table lists some of the more important viruses in each genus.

The present work is mainly dealing with two retrovirus genera, the γ-retroviruses and the lentiviruses. Further, the most important viruses in the respective groups are MuLV and HIV. In the following, these two groups and viruses will be described somewhat more in detail.

γ-retroviruses

The γ-retroviruses corresponds to the previous genus of mammalian C-type retroviruses. It is the most spread of the retroviruses genera, is found in many different species, and γ-retroviruses can be exogenous or endogenous. There are no known human exogenous γ-retroviruses, but endogenous proviruses exist [24, 212, 293, 294]. The number of primate exogenous γ-retroviruses is small; the best example is GaLV [53].

The γ-retroviruses are on the genomic level of the simple kind, i.e. they only contain the basic functional retroviral genes (Table 1). However, some
strains contain oncogenes, which are cellular genes picked up by the virus that act as tumour inducing factors; for review see [251, 290]. This is the case for several laboratory strains of MuLV [129].

The most studied γ-retrovirus is MuLV. A large number of MuLV strains have been described, of which many are endogenous and found in most strains of mice. They can be divided in groups depending on their ability to replicate in cells of different origin; for review see [129]. One example of endogenous MuLV is a virus found in the SP2/0 cell line [54, 142] used for producing MAbs [1, 222]. Exogenous strains of MuLV occur in nature but are not common [80]. The laboratory strain MMuLV, originally isolated from a spontaneous mouse tumour [165] is able to give rise to lymphoma when injected into new born mice of some strains. Like many other retroviruses it causes malignance by distortion of the cell functions by insertion into the host cell genome [33].

Table 2. Classification of exogenous retroviruses

<table>
<thead>
<tr>
<th>Genus</th>
<th>Morphology/ genome</th>
<th>Virus examples</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>C-type / simple</td>
<td>Avian leukosis virus (ALV)</td>
<td>Leukemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Avian myeloblastosis virus (AMV)</td>
<td>Myeloblastosis (oncogenic)</td>
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<td></td>
<td></td>
<td>Rous sarcoma virus (RSV)</td>
<td>Sarcoma (oncogenic)</td>
</tr>
<tr>
<td>β</td>
<td>B- or D-type / simple</td>
<td>Mouse mammary tumour virus (MMTV)</td>
<td>Breast tumours</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jaagsiekte sheep retrovirus (JSRV)</td>
<td>Lung tumours</td>
</tr>
<tr>
<td>γ</td>
<td>C-type / simple</td>
<td>Murine leukemia virus (MuLV)</td>
<td>T-cell lymphoma</td>
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<td></td>
<td></td>
<td>Feline leukemia virus (FeLV)</td>
<td>T-cell lymphoma, immunodeficiency</td>
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<tr>
<td></td>
<td></td>
<td>Gibbon ape leukemia virus (GaLV)</td>
<td>Lymphoma</td>
</tr>
<tr>
<td>δ</td>
<td>C-type / complex</td>
<td>Human T-lymphotropic (or T-cell leukemia) virus (HTLV) I &amp; II</td>
<td>T-cell leukemia, neurological disorders</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bovine leukemia virus (BLV)</td>
<td>B-cell lymphoma</td>
</tr>
<tr>
<td>ε</td>
<td>C-type / complex</td>
<td>Walleye* dermal sarcoma virus</td>
<td>Dermal sarcoma</td>
</tr>
<tr>
<td>Lenti</td>
<td>Lenti / complex</td>
<td>Human immunodeficiency virus (HIV), 1 &amp; 2</td>
<td>AIDS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Simian immunodeficiency virus (SIV)</td>
<td>Species dependent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Feline immunodeficiency virus (FIV)</td>
<td>AIDS-like disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Equine infectious anemia virus (EIAV)</td>
<td>Anemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Visna/maedi virus (VMV)</td>
<td>Lung and neurological disorders (sheep)</td>
</tr>
<tr>
<td>Spuma</td>
<td>Spuma / complex</td>
<td>Simian foamy virus</td>
<td>None known</td>
</tr>
</tbody>
</table>

*The walleye is a North American fresh water fish (Stizostedion vitreum) belonging to the perch family.
MuLV, the Moloney strain or other, is commonly used as a tool in research, e.g. as pseudotypes (mixed viruses) with HIV-1 [105, 252]. Vectors based on MuLV are used in gene therapy studies; for review see [146, 275]. Quantitation of MuLV in such systems is one of the possible applications of RT activity assays for MuLV. The RT of MMuLV has also been used for making cDNA [98].

A virus closely related to MuLV is FeLV, feline leukemia virus [115]. FeLV is widely spread in domestic cats throughout the world [70, 169, 300].

Also interesting for this work is the presence of endogenous γ-retroviruses in pigs. PERV obtained from the PK-15 cell line [246, 271], has the ability to infect human cells [190]. This is a major concern for the idea of xenotransplantations from pigs to humans; for review see [25, 46, 147, 229].

Lentiviruses

Overview

The most important lentiviruses are listed in Table 2, and a phylogenetic tree over the genus is shown in Fig. 3. So far the only known human lentiviruses are HIV-1 and -2, although SIV can infect humans under certain circumstances [124]. The fact that HIVs and SIV are genetically similar as well as their hosts indicates that they share a common origin [110, 208]. Many types of SIV do not cause disease in their natural hosts, but do so when infecting other species [96]. This phenomenon, known as zoonosis, is common in the virus world. It has been shown that HIV-1 probably originates from chimpanzees [74, 224], while HIV-2 came from sooty mangabeys [37, 106]. HIV-1 is believed to have been transferred to USA from Haiti, where it had come from somewhere on the African continent [289].

As mentioned earlier, there are lentiviruses known from several non primate species (Table 2). One such virus, important to this work (I, III), is FIV [192]. Although not very closely related to HIV (Fig. 3), it causes a similar disease in cats, which has made it interesting as an animal model [21, 120, 178, 295]. It has a broader tropism than HIV and is able to infect not only CD4 cells, but also CD8 cells, B cells and others [63]. In addition, it lacks the tat, vpr and vpu genes found in HIV-1. FIV infection is relatively widely spread among both domestic and wild cats in some areas [70, 169, 300], and males are more likely to be infected than females, probably because of spread through rivals biting each other. Although the RT of FIV only has about 40% sequence homology to that of HIV-1, it has very similar assay condition preferences to HIV-1 RT [21].
HIV and its infection

**Course of HIV disease**

For HIV infection to occur, the virus must first be transferred in an amount large enough to cause infection. The main routes of transfer are through blood or sexual contacts. Sexual transfer from a man is regarded as easier than from a woman, as semen contains virus to a similar degree as blood [92, 197]. Also, children are frequently infected from the mother, mainly at birth and through the milk [227]. Distribution of the NNRTI NVP to the mother at the time of labour has shown to decrease the risk for infection of the child [32, 258, 266].

Once inside the body, HIV and other primate lentiviruses have tropism for cells of the immune system that express the CD4 receptor, CD4 cells. These cells include T-helper cells, macrophages, and also some cells in the nervous system. The direct cause of AIDS is the depletion of CD4 T-cells. The depletion takes place by a complicated process involving the killing of CD4 cells [4, 41], in a delicate equilibrium with the production of new CD4 cells [29].

The first sign of HIV disease is often, but not always, a short period of influenza like symptoms, appearing a few weeks after infection. This period is characterized by high VL (virus amount in the blood) and decreasing CD4 cell number. After the period of acute infection the VL stabilizes at a lower

---

Figure 3. Phylogenetic tree over Lentiviruses, after [145]. SMM, sooty mangabey monkey; SYK, Sykes monkey; CPZ, chimpanzee; AGM, African green monkey; MND, mandrill.
level, usually a few thousand RNA copies/ml, for a considerable time. As the VL increases the CD4 cell number slowly decreases. The disease stage AIDS is eventually reached, often several years after infection. The time before onset of AIDS is very variable, and it is known that some patients have been infected for many years without significant symptoms. These patients are called long time non-progressors [187, 216]. During the period when the patient does not show clinical symptoms, the provirus is found in different cells in several reservoirs of the body. This is one reason why anti-HIV treatment is not able to eradicate the virus [223].

HIV-1 subtypes

HIV-1 is very diverse and is continuously mutating [110]. The presence of VL suppressing treatments of patients makes this diversity even larger, due to the selection for drug resistance. However, the diversity is still major for non-resistant virus, and it is believed that all HIV infected persons host a large genetic variation in their virus population [110].

HIV-1 has been divided into subtypes, depending on features in the gag and env genes. The major group of subtypes, assigned M, consists of subtypes labelled A to K. The division in subtypes is somewhat different depending on source; sometimes subtypes E and I are not listed [193, 267]. These types are geographically distributed in a rather distinct way [75, 186, 194, 267]. Since the emergence of the HIV epidemic, subtype B has been dominating in the Western world. Therefore many methods for e.g. VL determination were originally designed for subtype B, and have later been more or less adapted for detecting the other subtypes.

Also, mixed subtypes (recombinants) including typical parts of usually two subtypes frequently occur. For instance, recombinants between A and B are common in Russia and neighbouring countries [267]. Some RNA based VL methods have problems quantitating certain recombinants [5].

HIV-1 variants that do not belong to any of the group M subtypes have been assigned to belong to group O (Outlier) [93, 285]. Different isolates within the O-group differ as much from each other than between the subtypes of group M, so group O is not a subtype. Later, another group of viruses not belonging to M or O was assigned N (Non M, non O) [217, 250]. Both group O and N virus are relatively rare and mainly found in Cameroon and neighbouring countries. The established VL methods commonly have problems detecting group O and N viruses. There are differences between subtypes in response to anti-HIV drugs and the emergence of resistance; for review see [254].

HIV-2

The genome of HIV-2 differs from that of HIV-1 to a such extent that it is regarded as another virus. It is so far mainly found in West Africa, particularly Guinea-Bissau and neighbouring countries [26, 209].
The disease connected with HIV-2 infections is milder than for HIV-1 [152] and there are indications that it is less readily spread by heterosexual contacts [119]. The VL associated with HIV-2 is commonly lower than in HIV-1 infection [8, 240].

The drugs designed for HIV-1 have different capacities to inhibit HIV-2. The NRTIs are generally functional, while the NNRTIs are not [102]. This makes the treatment of HIV-2 more problematic. Also, the commercial VL monitoring techniques are not functional for HIV-2, due to the genomic differences.

The RT activity is, as mentioned, indispensable for the infectivity of the virus. RT is thus always present in an infectious virion, no matter which type or subtype the virus belongs to. This has made developed RT based tests for VL and DS particularly valuable for HIV-2.

RT enzymes and assays

Enzyme

Function

The virally encoded enzyme reverse transcriptase (RT) plays a fundamental role in the replication of all retroviruses. RT is responsible for the transcription of viral RNA into DNA, that is integrated in the genome of the host cell and subsequently giving rise to new virus particles. In the HIV-1 particle, somewhere around 80 copies of RT are reported to be packed inside the virus core [135].

All known RTs have three enzymatic functions: a) The RNA dependent DNA polymerase; b) The DNA dependent DNA polymerase; c) The ribonuclease H function.

The reverse transcription of the viral genome is a complex process including several steps and strand displacements. For more detailed descriptions of the process of reverse transcription, see general reviews [94, 269].

It starts with the annealing of the tRNA primer (for MMuLV tRNA Pro, for HIV-1 tRNA Lys) to a primer binding site close to the 5’end of the genome. From this point, a piece of minus-strand DNA is produced until the enzyme reaches the 5’ end. At the same time, RNase H degrades the original RNA that has been transcribed, acting in a position 16-18 nucleotides from the polymerising site.

When the 5’ end has been reached, the produced DNA strand anneals to the 3’end of the RNA, by the help of a repeat (R) segment found at both ends of the genome. The building of the minus-strand DNA then continues with the RNA as template, followed by partial RNase H degradation.

Thereafter, the minus-strand synthesis reaches a poly-purine tract (PPT) of the RNA. This is partially cut by RNase H in a specific way, yielding a
RNA/DNA duplex that acts as primer for the plus-strand DNA synthesis. This starts with the produced minus-strand DNA as template, the priming part of the tRNA is copied, the tRNA degraded by RNase H and the double stranded DNA is finalized (Fig. 1), with identical LTRs at both ends. The presence of two genomic RNA copies may be required for this process. RT does not have any proofreading function, thus the enzyme makes a lot of errors by incorporating incorrect bases [20]. This is one of the reasons for the high mutation rate of retroviruses [265].

All RTs are metal ion dependent for their activity, in the case of HIV-1 RT Mg$^{2+}$. The activity of MMuLV RT is generally known to be Mn$^{2+}$ dependent, however, the metal dependence can vary due to the nature of the template/primer [12, 15]. In the studies presented here, only prA as template and odT as primer have been utilized for MMuLV RT, and the data presented in I and the mentioned references suggest that the RT is strongly Mn$^{2+}$ dependent at the reaction conditions used.

Structure
The RT enzyme is special among DNA polymerising enzymes in that it can utilize both RNA and DNA as template. Although there are similarities in RT to other polymerases, this fact, and that RT lacks proofreading function, makes RTs unique in the DNA polymerase superfamily.

Most RTs, including that of MMuLV, are monomeric enzymes where all the functions are found in the same protein. It is cleaved by PR from the Pol/Gag protein, together with PR and IN. The overall structure of the enzyme, as determined by x-ray crystallography, is often described as a right hand, where the active site is located in a cleft of the “palm”, between the “fingers” and the “thumb”. The “hand” structure was first elucidated for HIV-1 RT [112, 127, 276] and has recently been solved also for the RT of MMuLV [49].

In the case of MMuLV, the 74kD protein may form a homodimer during DNA synthesis [268]. The RT of HIV-1, and of the other primate lentiviruses, is however a heterodimer, in the case of HIV-1 consisting of p66 and p51. The p66 subunit, which is the part with hand shape, comprises both the polymerase and RNase H domains of the RT. The p51 subunit corresponds closely to p66 but lacks the C-terminal RNase H region.

RT activity assays
Measurements of RT activity is the over all theme of this thesis. Therefore, a somewhat detailed overview of the history of RT assays could be motivated. The following is an attempt to illustrate the development of the methods of RT assays and the state of the art at the introduction of the methods involved in this thesis.
A historical overview of enzymatic RT assays, focusing on the RTs of MMuLV and HIV-1, is shown in Table 3. In the describing text the methods are referred to by the numbering in the first column of the table. Some of the mentioned papers also give results obtained for other DNA polymerising enzymes, which will not be discussed here. Also, with minor exceptions, no attempt will be made to compare the performance of the different methods in terms of sensitivity. The main reason for this is that the data in the different papers are presented in such diverse ways in terms of enzyme reaction times and other parameters, that it would be virtually impossible to reconstruct their sensitivities under comparable conditions. The methods are also hard to compare due to the use of test enzymes from different sources.

In most cases the described assays include washes between steps, which are not mentioned in the descriptions.

Radioactive

The traditional RT assays were based on incorporation of a radioactively labelled substrate into a soluble DNA strand, usually using a homopolymeric RNA strand as template and a complementary oligo DNA as primer. The activity of RT was proportional to the amount of DNA product precipitable by acid (e.g. on a filter membrane) and detectable by an appropriate technique. For a long time, all methods described were developed mainly for use with virus from culture supernatants.

Shortly after the discovery of RT, the template primer specificities were determined for the RTs of MMuLV (1). It was found that the reaction velocity was higher with prA/odT than with prC/odG. The original method with \(^3\)H was later adapted for use with \(^32\)P and a more automated procedure (3).

A solid phase reaction was introduced as early as in (2). Here, prA on agarose served as template for MMuLV RT, and the system was used for study of the interaction between the binding of RT to the template/primer and antiviral compounds (2), [161].

Template/primer studies for HIV-1 RT (4, 5) also found a higher activity using prA/odT, but interestingly in (5) with a preference for Mn\(^{2+}\) over Mg\(^{2+}\) when using prC/odG. The possibility to increase the assay sensitivity by use of prolonged assay time, an important feature in this thesis, was explored in (6). Here, the authors were able to keep the RT activity reasonably linear for up to 72 h.

The method described by members of our group in 1990 (7), introduced two important features. First, the substrate \(^{125}\)I-dUTP was used. This analogue to dTTP was nicely accepted by the RT, and due to its high specific radioactivity and efficient detection an increase in assay sensitivity was obtained. Second, the polyamine spermine was introduced as an assay component, becoming a crucial ingredient for the sensitivity of all our enzyme assays. The mentioned assay was later modified for use with beads and sepharose as solid matrices (8). Here, a purification step, in which the RT was
allowed to bind the template/primer followed by a wash, was introduced. The method was also used for determination of RTb-Ab [175].

A different concept for assay of RT, allowing the use of samples containing a lot of disturbing factors, was explored in (19). Here, MAbs against RT, which were able to bind the enzyme without affecting its activity, were immobilized on beads. The RT was then allowed to bind the MAbs, whereafter a reaction buffer containing prA/odT and ³H-dTTP was added. The enzyme reaction then took place with the RT on the bead, where the resulting RNA/DNA hybrid remained bound! The drawback of this type of assay is obviously its need for conserved sequences of the RT in order for the MAbs to bind. This method was later further adapted for non-radioactive use (24).

Very few RT assays optimized for MMuLV have been published since the pioneer days (1, 2, 3). An exception was (16) where (3) was adapted for use with 96 well plates, using ³H-dTTP as substrate.

Non-radioactive

There are several good reasons not to use radioactivity in enzyme assays. One is the obvious environmental hazards. A perhaps even more important reason is that use of radioactively labelled substrate normally results in a low assay sensitivity. Due to safety concerns and cost, the “hot” radiolabelled substrate is used at a low concentration, mixed with “cold” substrate. This means that only a minor fraction of the incorporated nucleotides are “hot”, resulting in poor sensitivity.

The first non radioactive RT assay published, (9) used a MAb against HIV-1 RT immobilized to a 96 well plate. The MAb was allowed to bind the sample RT, and the enzyme reaction took place in the well with prA/odT and, as substrate, BrdUTP. This dTTP analogue [87-89, 201] is similar to ¹²⁵I-dUTP and very well accepted by the RT. After the enzyme reaction, the double stranded product was hydrolysed using NaOH and the supernatant mixed with a HRP-labelled anti-BrdU MAb. The mixture was then transferred to another plate, on which anti-BrdU MAbs had been immobilized. After wash, the BrdU in DNA was quantitated using colorimetric detection. In the light of time, the method could be described as an early version of (24).

Immobilization of odT primer was introduced in (10), with prA added in the reaction buffer. Biotinylated dUTP (bio-dUTP), mixed with dTTP, was used as substrate in the RT reaction. Streptavidine conjugated to AP then bound the bio-dU in DNA, and was colorimetrically detected with pNPP. Interestingly, the AP reaction was stopped using NaOH, which prevented reading after prolonged time.
Table 3. Historical view over RT activity assays for MMuLV and HIV-1. R = Radioactive; NR = Non-radioactive; PCR = Involving PCR.

<table>
<thead>
<tr>
<th></th>
<th>Type</th>
<th>Solid matrix</th>
<th>Template</th>
<th>Primer</th>
<th>Labelled substrate</th>
<th>Detection</th>
<th>RT</th>
<th>Reference</th>
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<td>prC</td>
<td>odT&lt;sub&gt;14&lt;/sub&gt; odG&lt;sub&gt;14&lt;/sub&gt;</td>
<td>&lt;sup&gt;3&lt;/sup&gt;H-dTTP &lt;sup&gt;3&lt;/sup&gt;H-dGTP</td>
<td>β-count</td>
<td>MMuLV</td>
</tr>
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<td>prA</td>
<td></td>
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<td>β-count</td>
<td>MMuLV</td>
</tr>
<tr>
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<td>None</td>
<td>prA</td>
<td></td>
<td>odT</td>
<td>&lt;sup&gt;3&lt;/sup&gt;P-dTTP</td>
<td>β-count</td>
<td>MMuLV</td>
</tr>
<tr>
<td>4</td>
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<td>prA</td>
<td></td>
<td>odT&lt;sub&gt;12-18&lt;/sub&gt; odG&lt;sub&gt;12-18&lt;/sub&gt;</td>
<td>&lt;sup&gt;3&lt;/sup&gt;H-dTTP &lt;sup&gt;3&lt;/sup&gt;H-dGTP</td>
<td>β-count</td>
<td>HIV-1</td>
</tr>
<tr>
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<td>prA</td>
<td>prC</td>
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<td>HIV-1</td>
</tr>
<tr>
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<td></td>
<td>odT&lt;sub&gt;10&lt;/sub&gt;</td>
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<tr>
<td>21</td>
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<td>MMuLV</td>
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<td>[179]</td>
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<td>BrdUTP</td>
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<td>HIV-1 IV</td>
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</table>
The assay (11) included three substrates! Dig-dUTP was the main labelled substrate, mixed with dTTP and small amounts of bio-dUTP. The RT reaction was allowed to take place in solution, and the mixture was transferred to a strav-coated plate where the product was immobilized. The same product was colorimetrically detected using HRP-conjugated anti-dig Abs. This method was made into a commercial kit from Boehringer Mannheim.

In 1993, Suzuki and co-workers published several versions of their non-radioactive RT assays. The first version used beads as solid phase (12), and was otherwise similar to (11). The second (13) used bio-odT as primer, and dig-dUTP as substrate. After reaction in solution, the product was bound to biotin on a plate and detected using AP-anti-dig-Ab and a chemiluminescent substrate. The third version (14) was the most interesting, since it utilized prA immobilized on a plate. Bio-dUTP was substrate, once again together with dTTP, and was by the enzyme built into DNA from an odT primer. Finally, HRP-strav and a colorimetric reaction was used. This method is the one previously published that is most similar to the assays used in this thesis. It should, however, be noted that for method (14) short assay times were suggested, both for the RT and the HRP reactions.

The assays involved in this thesis (20, 22, 23, 27, 28) will be discussed in the Thesis work section. To my knowledge, the only non-radioactive RT assays for MMuLV without PCR amplification that have been published are (22) and (23).

Another assay to mention from our group is (24). This method was a colorimetric adaptation of (19). Here, MAbs were immobilized on plates instead of beads, and BrdUTP and AP was used in a similar way as in (20). The method was useful for crude samples, e.g. plasma, however with a detection limit far from that obtained with the ExaVir Load assay (III, IV).

The method (26) is a further improvement of method (10). The same type of solid phase RT reaction is used, but the assay sensitivity is improved by several alterations. These include an RT purification step similar to that in (7), product digestion by DNase and a chemiluminescent detection system. The authors claim an assay sensitivity similar to that of EVLv1 (III), and that the method is able to measure RT activity directly in plasma samples. They do, however, not show any data from testing of samples from HIV-1 infected persons. The method is now also a kit from Asahi Kasei Pharma.

Finally, EVLv1 and 2 (27, 28) should be included in this listing, as the RT assay parts of these tests have very high sensitivity and in principle could be used also without the separation procedure.

Methods involving PCR
Since the invention of PCR [171] this very powerful method has been the major tool for many purposes of medicine and biology, including such diverse fields as HIV diagnostics and forensics. The wish for ultra high sensitivity has resulted in the development of several RT assay methods involving
PCR \((15, 17, 18, 21, 25)\). They are all based on an RT reaction in soluble phase, using a variable template and a complementary primer. The product is then amplified by PCR, and finally detected. The important differences between the methods are mostly found in this detection step.

The first presented procedure of this kind, that by Silver et al \((15)\), utilized a two phase system. The RT reaction took place as the upper phase in a tube, with a template from Brome Mosaic Virus (BMV) and a complementary primer. The PCR components were in the lower phase of the tube, with a layer of wax between. After a short RT step, the PCR was started with high temperature, leading to the melting of the wax. After PCR, a \(^{32}\)P-labelled oligonucleotide was hybridized to the product by further amplification cycles. Finally, the mixture was separated by electrophoresis and the radiolabel detected by autoradiography. The method was reported to work for several RTs, and to detect activity corresponding to only a few virions of HIV-1.

The PERT assay \((17)\) was in most respects similar to \((15)\). It used the RNA of bacteriophage MS2 as template. The product after PCR amplification was detected using a \(^{32}\)P labelled probe in Southern blot, and x-ray film. Also a non-radioactive detection system using plates and dig- and bio-labelled probes was used. The assay claims a similar sensitivity as \((15)\). Both \((15)\) and \((17)\) used the same assay conditions for the RTs of HIV-1 and MMuLV.

The Amp-RT assay \((18)\) was very similar to \((17)\) but used a different template, the RNA of the encephalomyocarditis virus (EMCV). In the original version, this assay used radioactive detection after electrophoresis of the PCR product. The PERT and Amp-RT assays have both later been adapted for VL determinations, and Amp-RT also for DS.

In work \((21)\) the authors compared the assay variants \((15, 17, 18)\) for MMuLV RT, using their respective template/primer systems. It was found that backgrounds due to RT-like activity from the thermostable polymerase used in the PCR step could be eliminated by changing this polymerase and introducing an RNAse digestion step after the RT reaction.

Finally, the assay according to \((15)\) was modified in \((25)\) with the use of a fluorescent detection system. The assay was able to detect 10 molecules of MMuLV RT, and was used to test several supernatants from different cell lines, e.g. finding RT activity in SP2/0 supernatant as in \textbf{I}.

The PCR based RT assays are extremely sensitive and are able to detect minute amount of RT in almost any sample. However, drawbacks of these methods are obviously the need for PCR equipment and facilities, making these assays costly and less useful for routine work. Another problem is the sensitivity to disturbances in the samples. The methods can be used for VL and DS determinations, but the mentioned drawbacks are even more pronounced for these applications.
RT activity blocking antibodies

Most RTs are immunogenic in the host organism, and give rise to an antibody response. Some of the Abs produced have the ability to block the activity of the enzyme [225]. This feature is for RT activity determinations mainly a problem, but it can also be used for several purposes. One is diagnosis, by measurement of the presence of RTb-Ab in e.g. the blood of a potentially HIV infected individual. The decrease of RTb-Ab in HIV patients have also been shown to be connected to progression to late stages of disease [176].

Another purpose of RTb-Ab measurement is to study the relationships between different RT isozymes. RTb-Ab found in an infected animal is in many cases able to block the RT activity, not only of the same virus but also of related viruses, based on differences in the primary sequence and the enzyme structure. This facts can be used for phylogenetic mapping of the viruses. For instance, in [246] the cross reaction between RTb-Ab against MuLV and FeLV was demonstrated. Several studies have been carried out to compare the ability of RTb-Ab against HIV-1, HIV-2 and SIV RT to block the other enzymes. Some studies, as [278], found RTb-Ab to these viruses to be type specific, while others have shown that RTb-Ab against SIV can block HIV-2 RT well but HIV-1 RT poorly [61] or that RTb-Ab against HIV-2 can block HIV-1 RT but not the other way around [11]. According to unpublished data from our group, RTb-Abs against different HIV subtypes show a complex pattern of inhibition of other subtypes, which was also the case for RT activity blocking MAbs made against HIV-1 RT of subtype B [222]. There are also unpublished data from our group indicating that some cross reactivity may occur between RTb-Ab against FIV and HIV-2/SIV.

Diagnosis and monitoring of HIV infection

Diagnosis

The standard way to determine an HIV infection is by the detection of antibodies against the virus. This test is performed by ELISA, where the ability of plasma or serum to bind viral lysate or proteins is determined.

To confirm that the person is infected a western blot, or the similar RIBA test (Chiron) [126] could be performed. Here, the reactivity of the persons antibodies against the different virus proteins is determined after separation of the viral proteins by electrophoresis. In a positive test the patient serum should react with two or more of p24, gp41, gp120 or gp160.

RTb-Ab can, as mentioned, also be used for diagnosis, although this has not become a procedure included in the standard set of tests. p24 antigen, by the Schüpbach method, may also be useful for diagnosis, particularly in paediatric samples [245].
CD4 cell count
The number of CD4 positive T-cells (CD4 cell number) is regarded as the most important parameter for determining the status of an HIV patient, as it directly reflects the stage of disease. During HIV infection, the number of CD4 cells in the blood stream decreases. A normal value of CD4 cell number in a healthy person is between 500 and 1500 cells/µl. An HIV infected person with a value below 200 CD4 cells/µl is regarded as having reached the stage of AIDS. When this happens, a patient in the developed world is normally treated. However, in the developing world treatment is so far not common enough.

The CD4 cells are most commonly counted using flow cytometry, but other methods are also available; for review see [45, 83].

Viral load
Although CD4 cell count remains important for monitoring HIV infection, the measurement of VL (amount of virus per volume of patient plasma) has become more important with the use of anti-HIV treatment. If the virus becomes resistant, this will be more rapidly seen in the VL than in CD4 cell number, as the process of CD4 cell decline is relatively slow compared to VL development [158]. A higher VL results in a higher rate of infection of CD4 cells, leading to a more rapid disease progression.

Therefore, in order to get information about the status of the patient, VL is important to measure. Also, VL has been observed to be the most reliable parameter for patient prognosis, giving better information about the risk for the patient to develop AIDS within a certain time [157, 158]. Ideally, both CD4 cell count and VL should be used for assessing patient prognosis, particularly during therapy [154].

Although the definition of VL is (or should be) the number of infectious HIV virions present per ml of patient plasma, VL is in most cases given as RNA copies/ml. The reason is that RNA based methods are most commonly used. For the p24 based methods, VL is given as pg Ag/ml. For the RT based methods, the RT activity could be expressed in different ways, in the case of ExaVir Load as fg RT/ml plasma.

RNA based
The Roche Amplicor RT-PCR test for HIV-1 RNA [170] is widely used in the developed world and has become the gold standard method for VL determinations. After reverse transcription, a region of the gag gene is amplified by PCR, and quantitated using an internal standard, hybridization to specific probes and finally colorimetric detection. The current version of Amplicor is called 1.5; it has replaced the 1.0 version and uses an improved primer mixture giving a better detection of non-B subtypes [2, 3, 103, 109,
Also, a standard and an ultra sensitive version [259] exist, the latter using a larger amount of plasma and concentration of virus by centrifugation. Further, both manual and automated (Cobas) versions are in use. The results from Amplicor are obtained within about 6 h.

Two competing RNA quantitation methods exist, which are, as Amplicor, approved by the FDA. These are the nucleic acid sequence-based amplification, NASBA (NucliSens, from bioMerieux), and bDNA (Versant, from Bayer). For general reviews on these methods, see [195, 230].

NucliSens NASBA [125, 284] is a complicated procedure, reminding of PCR but not including any heating/cooling cycles. It involves three enzymes (AMV RT, T7 RNA polymerase, RNase H), and is likewise based on amplification of gag. NucliSens utilizes three internal standards, and the final product is bound to magnetic beads and detected with a chemoluminiscent system. Also NucliSens has been developed in an ultra sensitive version, able to detect 80 RNA copies/ml. The results take about 8 h to produce.

Versant bDNA [279], currently at version 3.0, is based on enhancement of the signal rather than the RNA. The RNA in lysate from pelleted virions of the sample is bound to pol-specific probes on the wall of a plate. Further probes, which are able to bind more AP-conjugated probes are added, and the AP is detected by chemoluminscence. An external standard curve is used for quantitation. Also this method is available in an ultrasensitive version at 50 copies/ml [43]. As the assay includes an overnight incubation, 24 h are required for results.

A number of studies have been carried out to compare these methods. The overall findings are that the performances of the current versions are comparable in most respects. However, Amplicor is reported to give higher VL values than Versant [101, 172], not least for non-B subtypes [62]. NucliSens is reported to underestimate some subtypes and certain recombinants [5, 10, 86, 113]. Amplicor 1.5 and Versant 3.0 both quantitate M subtypes well [10, 113], but detect group O samples poorly [264]. None of the commercially available RNA based methods is functional for HIV-2, but experimental methods are being used [8, 220].

There are also new RNA based methods, which have been tested in studies but are not yet fully commercially available. These include the LCx HIV RNA assay from Abbott, tested in several studies [6, 22, 51, 116, 122, 198, 264]. It was found to detect all tested subtypes including group O [116, 198, 264] but, on the other hand, to have problems quantitating some HIV-1 recombinants [6]. Also, real time PCR methods have been developed, which use fluorescent probes for their real time detection [81, 91, 184]. One of these methods [184] was able to detect less than one RNA copy/ml, when virus in 7 ml plasma was concentrated by centrifugation.
**Antigen based**
The p24 (CA) Ag is a common marker for HIV and widely used for quantitation of HIV in cell culture. The assay is normally based on coupling of p24-directed Abs to a plate, binding of p24 to the Abs and detection by use of a second Ab conjugated to an enzyme for colorimetric detection. An example is the method by [260], which in II was compared to RT for quantitation of HIV in cultures. Different kits have also been commercially available, like the one from Abbott also used in II, and kits from DuPont and Perkin-Elmer, used for p24 capture in VL methods [35, 173, 214].

There are, however, principal problems with p24 as a marker for HIV. One is that the genetic variation of the virus decides the specificity of the assay, depending on which type of virus the used p24 directed Abs were produced against [100]. Another is that the presence of p24 is not connected to the infectivity of the virus.

For VL determinations p24 has been used for some time, mainly in form of the method developed by Schüpbach; for review see [234, 236]. He and co-workers started with detecting HTLV in patients by measuring p24 [231], and continued to do so in HIV-1 patients. The problem with p24 complexed by p24-binding Abs [191] was solved by boiling of the sample [232]. Further developed versions, including a detection step boosted by a commercial signal amplification kit [233, 234], have been compared to RNA based methods in several studies and shown to correlate with these [28, 34, 35, 173, 188, 214, 235]. It has also been found useful for e.g. diagnosis of infected infants [245], and shown to give prognostic information [235, 257]. The advantages of this method are similar to those of EVL, including relative simplicity, low cost and ability to detect non M subtypes [214]. The method is considerably faster than EVL.

However, problems with p24 for VL monitoring still remain. The correlation between p24 and RNA is often not very strong [28, 34, 173, 188] and the sensitivity is sometimes doubtful, as occasionally samples with >100 000 RNA copies/ml are not detected [188, 205].

**RT based**
A few studies using the Amp-RT method [99] for VL determination based on RT activity have been published [77, 78, 210, 240]. Also, the very similar PERT assay has been utilized for this purpose [34].

In [78] the correlation between RT and RNA was found to be reasonably strong (r=0.73) for samples from patients at various stages of HIV-1 infection. RT activity and RNA covaried nicely for several patients tested over time. In [210], Amp-RT was shown useful for monitoring infants, also with a decent correlation (r=0.63) to NucliSens. The studies [77] and [240] used Amp-RT to study group O and HIV-2 infections, respectively. The earlier study showed that RT activity indicated treatment failure, the latter that VL appeared to be lower in HIV-2 than HIV-1 infection. These studies are ex-
amples of RT activity as a functional tool for VL determinations in cases where RNA based methods do not work.

Although Amp-RT and PERT are very sensitive assays, there are problems with these methods for VL determinations. One is that the assays are sensitive to RTb-Ab. Attempts have been made to compensate for this by assessing the effect of RTb-Ab by assaying the sample together with recombinant enzyme [34, 78]. However, some samples did because of this not give any useful VL values [34]. A problem with the mentioned kind of test for inhibition is that the added control enzyme may not be blocked by the RTb-Ab in the sample to the same extent as the internal enzyme, due to antigenic differences. Obviously, the need for PCR facilities is also a problem if these methods are to be used in resource limited settings, and so far these methods have only been used experimentally. The use of Amp-RT for DS determinations has also been explored, as described below.

Principles compared
All the three discussed principles for VL measurement (RNA, p24 and RT) have been compared in one study [34]. Here, the three commercial RNA based methods (version 1.5 of Amplicor, 2.0 of Versant) were tested together with p24 and PERT. Of the 103 samples tested, 55 were positive in the most sensitive test (Amplicor). All other methods were in the study compared to Amplicor, so the pairwise relations between the other methods can not be deduced from the paper. The results were, in very brief, that Amplicor was the most sensitive method, followed by, in order, PERT, p24, NucliSens, Versant. All methods except p24 had correlations to Amplicor with r>0.7, while p24 only correlated with r=0.4. An included O isolate was detected only by PERT and p24. Although the PERT assay had severe problems with RTb-Ab, the study suggests RT as a competitive marker for VL.

The relationship between RT and RNA is an interesting issue mentioned in III and IV. Garcia-Lerma and co-workers [78] found that for the mentioned patients (previous section) the RT/RNA ratios remained relatively constant over time, while the differences between patients were significant. The authors suggest that this may be due to individual differences in the amount of functional RT in the virions, possibly in connection to other phenotypic differences between patient virus, such as tropism.

Further, a recently published study [153] suggests RT as a more valid marker for infectious virus particles than RNA or p24. The authors compared the ability of these markers to determine the actual number of infectious virions in culture supernatants. The results showed that RT activity was the best marker. As the information estimated by VL tests is the amount of infectious particles, and the results tell that this is reflected by RT activity, the study suggests the usefulness of RT as marker for VL. As previous studies suggest that only about 1% of virions in an infected person are actually infections [56], this should not be less valid when virions have been purified from plasma prior to activity measurement, which is the case for EVL.
HIV therapy and drug resistance

Chemical therapy against HIV-1 was introduced in 1987; the first functional drug was AZT. So far eight NRTIs, three NNRTIs, eight PIs and one fusion inhibitor have been approved by the FDA for use in the USA (Table 4). All these drugs have in common that they block the replication of the virus and thus slow down the course of the disease. None of them is however able to eradicate the virus, and when treatment stops or resistance occurs the VL starts rising again. All drugs give side effects to the patients and many are also significantly toxic. Recommendations for the use of anti-HIV drugs are regularly issued and updated [299].

Table 4. Anti-HIV drugs approved in the USA by the FDA.

<table>
<thead>
<tr>
<th>NRTIs</th>
<th>NNRTIs</th>
<th>PIs</th>
<th>Fusion inhibitor</th>
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<tbody>
<tr>
<td>zidovudine (AZT)</td>
<td>nevirapine (NVP)</td>
<td>ritonavir</td>
<td>enfuvirtide (T-20)</td>
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<tr>
<td>lamivudine (3TC)</td>
<td>efavirenz (EFV)</td>
<td>indinavir</td>
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<tr>
<td>didanosine (ddI)</td>
<td>delavirdine (DLV)</td>
<td>saquinavir</td>
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<tr>
<td>zalcitabine (ddC)</td>
<td></td>
<td>nelfinavir</td>
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<tr>
<td>stavudine (d4T)</td>
<td></td>
<td>amprenavir</td>
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<tr>
<td>abacavir (ABC)</td>
<td></td>
<td>lopinavir</td>
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<tr>
<td>emtricitabine (FTC)</td>
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<td>atazanavir</td>
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<td>tenofovir (TDF)</td>
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<td>fosamprenavir</td>
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Anti-HIV drugs

RT directed

Although the field of targets for therapy against HIV is growing, the RT directed drugs comprise an important part of all therapies. The nucleoside inhibitor AZT (azidothymidine, zidovudine) was the first drug to be approved for therapy against HIV-1. This analogue to thymidine has the ability to be inserted in the growing DNA by the RT. After this, however, it acts as a chain terminator as a new base can not be inserted next to it. Several more NRTIs have been introduced after AZT, and they all have this chain terminating function in common. Their character as analogues mean that they are recognized as normal bases by the RT, and thus they act as competitors to the real bases. All the NRTIs (and tenofovir, that is in fact a nucleotide analogue) are prodrugs that need to be phosphorylated to triphosphate form by host enzymes to become active.

While the NRTIs act after being incorporated into DNA by the RT, the NNRTIs inhibit the RT in the step before in a non competitive manner. They block the activity of the RT by binding into a hydrophobic pocket, located close to the active site of the enzyme. The steric interaction of the NNRTI makes the RT less flexible and thereby prevents it from further action [65, 253].
The NRTIs are generally functional for all types of HIV, and for many other retroviruses. However, HIV-2 and many group O HIV-1 are not sensitive to the current NNRTIs [55, 102, 211].

**Protease directed**

The introduction of the first PI in 1995 (saquinavir) meant a revolution for the anti-HIV treatment. More than one target now could be attacked, leading to a more reliable combination therapy.

All the approved PIs are peptides with a similar mechanism of action. They bind to a crucial aspartic acid residue at the active site of the enzyme [164] and thus prevent the cleaving of precursor proteins [68]. The search for new PIs is continuously developing by the help of structure design methods [13, 297].

**Other**

The only approved non anti-RT or -PI drug so far is enfuvirtide (ENF, T-20) [71]. T-20 is a fusion inhibitor, consisting of a peptide of 36 amino acids that interacts with gp41 (TM) after the binding of the virus to the cell. The use of T-20 has so far been limited, partially because it is distributed by injection, and it is regarded as a last resort, so called salvage therapy, for patients with a multi-drug resistance history. New possible targets for HIV drugs are continuously being explored, these include the IN enzyme and the RT priming.

**Combination therapy**

The idea of combination therapy is to improve the effect by attacking the virus in several ways at the same time. The purpose is also to make it more difficult for the virus to become resistant, in the hope that the amount of mutations needed would make the virus unable to replicate. So far the latter has not been achieved, but the hope for this increases by the introduction of new drug targets. However, the big success of combination therapy is its effect to maintain low virus levels and therefore making it much less likely that multidrug resistance will have a chance to occur.

Nowadays, due to the rapid emergence of resistance, monotherapy is very seldom used. Different combination variants are in use, normally including two NRTIs (often AZT combined with 3TC) and a PI or NNRTI, although some drugs should not be combined with each other [299]. The simultaneous distribution of several therapies has led to a much more effective treatment and thus an improved clinical situation for most HIV patients in the developed world [134, 162, 203]. It also leads to more complex patterns concerning resistance mutations, and problems with interpreting results from genotypic drug resistance assays.
Resistance to anti-HIV drugs

Resistance mutations and mechanisms

If the VL in a patient undergoing treatment starts to rise, this is normally a symptom of emerging resistance of the virus. This is the consequence of mutations in the viral genome allowing the virus to circumvent the inhibitory function of the drug. This is a consequence of the high mutation rate of the virus, and the great genetic variation of the virus population in the infected person. Under continued drug pressure, more mutations accumulate with time, but when the treatment stops, VL will increase and wildtype virus takes over due to its greater replicative potential [52]. Viral resistance has been reported for all the HIV-1 inhibitors currently in clinical use. General reviews on HIV-1 drug resistance are found in [40, 239].

A mutation giving resistance towards one drug could result in regained sensitivity towards another. As a whole, the patterns of resistance mutations are very complex, and becomes even more so with time as new drugs and drug combinations are being used.

An increasing problem is infection with already resistant virus. In a recent study [292], 8.3% of newly diagnosed patients in US cities carried virus with resistance mutations. This underlines the need for resistance testing of newly diagnosed patients, in order to assure that a treatment to be introduced is functional.

The mechanisms of resistance to RT directed drugs are quite different for NRTIs and NNRTIs. Resistance towards NRTIs occurs by two mechanisms. One, direct binding discrimination, is based on RT mutations located close to the nucleotide binding site, allowing the enzyme to avoid incorporating the NRTI [133]. Examples of this are the substitutions Q151M, causing resistance to most NRTIs, and M184V, giving high level 3TC resistance. The other mechanism, nucleotide excision, is based on mutations allowing the RT to remove incorporated NRTIs from the DNA [138, 139, 159]. These mutations include substitutions in positions 41, 67, 70, 210, 215 and 219, and 69 insertions, all causing resistance to most NRTIs, particularly AZT. The reaction is ATP-dependent, as ATP provides the energy necessary for the excision of the NRTI. This resistance can be decreased by certain other mutations, such as the common substitution M184V mentioned above [95].

Resistance to NNRTIs is mediated by mutations in the binding pocket of the RT, making the NNRTI unable to bind and thus inhibit the enzyme. Several mutations, e.g. K103N and Y181C, give rise to resistance against all the used NNRTIs.

Assays for drug resistance

The methods used for determinations of HIV drug resistance are either genotypic of phenotypic, the genotypic being the cheaper and faster alternative. The genotypic way to determine resistance is to search the pol gene for mut-
tations known to be associated with reduced drug susceptibility [239]. This is done by PCR amplification of the gene, usually followed by DNA sequencing. There are several sequencing techniques, which are beyond the scope of this thesis; for review see [239]. Commercial methods include TrueGene, from Visible Genetics, and ViroSeq, from Applied Biosystems.

The main problem with genotypic resistance assays is the interpretation. All interpretations of genotypic data are based on phenotypic data about which mutations that give rise to which resistance. Although the presence of resistance mutations sometimes are directly connected to resistance, mutations may interfere with each other in ways that are hard to predict. One way to address the interpretation problems is the use of “virtual phenotype”, used as a brand name by Virco. This approach is to make a genotypic resistance test, and then compare the obtained resistance pattern with results within a database consisting of many matched sets of geno- and pheno-types. In this way, the phenotype connected to a certain genotype could be assessed based on previous results. This approach has been shown to predict treatment outcome as well as real phenotypic tests [226].

The phenotypic assays are based on determination of the ability of virus representing the patient’s phenotype to multiply in culture together with the drug. The classical approach was to isolate virus from patient cells, and infect a cell culture with these in presence of various concentrations of the drug and estimate the ability of the cultures to produce virus [114]. The amount of virus could be measured by RT or p24. Except for the very long time required for such an assay, this handling is prone to introduce artefacts from the virus propagation, such as selection of irrelevant virus due to the artificial conditions in the culture.

Later, recombinant methods have been developed [123, 196, 247], of which two are commercially available (Antivirogram, from Virco; and PhenoSense, from ViroLogic). Both are based on PCR amplification of the patient pol gene (RT and PR) and creation of a recombinant virus including the gene. This recombinant virus is then assayed for drug susceptibility in culture. The results of the phenotypic assays are given as fold increase in IC$_{50}$ compared to reference recombinant wildtype virus. The limitations of the current phenotypic assays are the lack of consensus of what fold increase in IC$_{50}$ that is clinically significant for a certain drug, as well as cost and the long time required.

Besides the established phenotypic assays, resistance to RT directed drugs could be determined by direct analysis of the RT. This has, before ExaVir Drug (V), been done using the PCR based Amp-RT assay [99], by assay of the RT in presence and absence of the drug [76, 79, 207, 291], in a similar way as in V. Amp-RT has so far only been used experimentally and does not seem to be planned for introduction as a clinical routine tool.
THESIS WORK

Aims

- To optimize and apply a simple and sensitive assay for the RT of MMuLV and other γ-retroviruses (I)
- To evaluate the ability of RT activity assay and p24 antigen to quantitate HIV-1 of different subtypes (II)
- To develop a technique for purification of HIV RT from patient plasma, useful for determination of viral load (III, IV)
- To utilize the purified RT for testing of susceptibility towards RT directed antiviral drugs (V)

The RT assays for HIV-1 and MMuLV

The RT assays involved in this thesis were based on an earlier assay version using beads as solid phase [90], and further developed to create a system that was sensitive and simple to use (Table 4). The 96 well plate concept is well established, and used for a multitude of applications in several fields of science. Due to this, equipment for use with this plate format, such as readers for colorimetry or fluorimetry, are found in many laboratories throughout the world. This fact suggests that the 96 well plate is the format of choice for methods supposed to be marketed for use in research as well as clinical applications.

Different modifications of the same assay system for RT activity have been used in all work included in this thesis. In I and II, the original Lenti RT kit [60] was used, in I together with the C-type RT kit described in that paper. In the remaining papers (III, IV, V) modified versions of the HS-Lenti kit [131, 148] were used for VL and DS determinations. An attempt to compare the assays involved is illustrated in Table 5.

The Lenti RT kit has been utilized in several studies for determination of e.g. RTb-Ab and mode of action of antiretroviral drugs [11, 61, 241, 242, 244], and as HIV quantitation tool in several studies by other groups.
Assay for HIV-1 RT and general features

The original Lenti-RT assay was presented by our group in the middle of the 1990’s. It was the result of a long development work, which is described in detail in [60]. The C-type RT assay (I) is based on the Lenti-RT assay. Here will be given a brief description of the most important features and components of the assays. The assay principle is visualized in Fig. 4.

Figure 4. The principle of the current RT assays (drawing by Kajsa Löwenhielm).

Assay plates and template/primer

Two different types of plastic 96 well plates are used in the methods presented here. Both these plates are commercially available from Nunc, Denmark. In the Lenti and C-type RT kits (I, II) the CovaLink plate is used, while in HS-Lenti, HS-Mn and the ExaVir kits (III, IV, V) the plate used is NucleoLink.

The most common template for RT activity assays is prA, with odT as primer. This template/primer combination is known to give a higher reaction velocity than prC/odG for some RTs [15]. Also, poly-purines are less sensitive to degradation by RNases than pyrimidine containing polymers. In our case, the prA/odT system was the first choice, as we had previously explored this [90, 174], and further that the substrate analogue BrdUTP could be used [202]. The prA template was immobilized to the CovaLink plate by a standard method, and the amount of odT primer optimized empirically [60].

After the publishing of I and II, the work with improving our RT assays continued. A new assay plate was developed, using the NucleoLink plate, on which the prA template was coupled using a solution in 1-methyl-imidazole [131]. The reaction conditions for both HIV-1 and MMuLV RT were reoptimized for use with this new plate. The conditions were however not al-
tered, except that the amount of odT primer was changed from 4 to 12 ng/well [131, 148]. The new RT activity kits were called the HS-Mn and HS-Lenti RT activity kits; the latter will be discussed in connection with III. The strongly increased sensitivity of the assay using the NucleoLink compared to the CovaLink plate (Table 5) is mainly explained by the ability of the NucleoLink plate to immobilize a larger amount of prA. The detailed chemistry behind the coupling of the template is not fully understood.

Other assay components
As mentioned, the dTTP analogue BrdUTP was used as substrate for the current assays. This analogue has similar chemical properties as the previously used IdUTP and has been shown to be equally well accepted by RT as dTTP [60].

Concerning the other components of the reaction buffer [60], (I), they will be briefly presented here. As buffer substance, HEPES has mainly been used in our assays, in both the Lenti and C-type assays at pH 7.6. The metal ion, necessary for the activity of all RTs, is Mg$^{2+}$ for HIV-1 and Mn$^{2+}$ for MMuLV RT. As chelator, to bind unwanted metal ions, EGTA or EDTA is included. Polyamine, spermine and/or spermidine, has shown to be a component crucial for the high sensitivity of our assays. Dextran sulphate is an inhibitor of the RNase H function of HIV-1 RT [163]. It has been shown to increase the time linearity of HIV-1 RT activity, but the activity of MMuLV RT is inhibited by dextran sulphate, and therefore the concentration was lowered in that assay (I). BSA is added as a carrier protein, and GTP to protect the substrate BrdUTP from degrading enzymes potentially present in the sample. The detergent Triton X-100 is included to lyse virus particles added in the sample, to make the RT available for reaction. Finally, a reducing agent was introduced in the assay for MMuLV RT, to prevent oxidation of thiol groups in cysteine residues [19].

Detection system
After the RT assay step, the plate is washed to remove sample, substrate and all other assay components. The wash buffer is slightly basic and contains Triton X-100. Normally, the wash has been done using four consecutive plastic buckets containing at least 0.5 l of wash buffer per plate. However, in I and II an automatic plate washer was used. This washer was later abandoned, as it did not provide a wash efficient enough for keeping the backgrounds satisfactory low and reproducible for VL or DS determinations. Lately, after the finishing of the work presented here, a new automated washing procedure for ExaVir has been optimized.

A commercially available MAb towards BrdU in DNA was used in all studies in this thesis. It is conjugated to alkaline phosphatase (AP), an enzyme that is used in several detection systems. The MAb is added to the plate in a buffer at basic pH containing non-fat milk as protector of the MAb.
[131]. After another incubation, allowing the MAb to bind the DNA, the plate is washed again. The AP could finally be detected by colorimetry, fluorescence of luminescence. In our assays, mainly colorimetry has been used, although the use of fluorescence has been explored in order to increase the sensitivity (III, IV). The substrate pNPP, added in a buffer at pH 10, gives a yellow colour reaction, measured at 405 nm. For fluorimetry, MUP (4-methylumbelliferyl phosphate) is used as substrate.

Assay for MMuLV RT (I)

The C-type RT assay for MMuLV was originally developed to provide a reliable non-radioactive assay kit for the detection of MuLV RT. The idea was that the kit would be useful for quantitation of MuLV and other γ-retroviruses in culture, e.g. during propagation of virus for use as retroviral vectors.

The optimization of the assay for the RT of MMuLV was performed using commercially available recombinant enzyme. The same plate and the same amount of odT primer and substrate (BrdUTP) as in the Lenti assay was used, as there was a wish that the bottle containing primer and substrate produced for the Lenti kit should be possible to use also in the new assay. The same was the case for the entire detection system.

During the development of the C-type RT assay it was noted that the recombinant MMuLV RT did not seem to have any detectable activity in the Lenti RT assay. This was consistent with previous observations, that some RT assays optimized for HIV RT did not work for MMuLV RT [111]. However, by replacing Mg$^{2+}$ for Mn$^{2+}$ and including the reducing agent β-mercaptoethanol the activity was strongly enhanced, while the activity of HIV-1 RT decreased. Final optimization resulted in a further greatly increased activity of MMuLV RT (I, Fig. 1). The assay was linear with time for about 22 h, and showed a linear relationship with MMuLV RT amount over a wide range (I, Fig. 2).

The presence of a reducing agent was needed in the assay for MMuLV RT, particularly for obtaining a good linearity with time. Such agents, most commonly DTE, are included in many enzyme assays. In our case, DTE was found to give high assay backgrounds, and therefore β-mercaptoethanol was originally used (I). In the commercial version of the C-type RT activity kit, β-mercaptoethanol was replaced by glutathione, giving the same positive effect but possible to provide as a lyophilised reaction component.

The described C-type and the later developed HS-Mn RT kits have been utilized in several published studies on PERV [47, 64, 130, 177, 181, 228, 237, 238, 274, 282, 298], MuLV pseudotyped with HIV or HTLV [117, 256], or spuma virus [36, 104, 218].
Retrovirus identification by the use of RT assays (I)

HIV-1 RT had a 16 fold higher activity in the Lenti assay compared to in the final C-type assay (I, Fig. 1). This specificity made us realize that the two RT kits could possibly be used for distinguishing different RTs from each other. For this purpose several RTs from different retroviruses were tested for their activities in the two assays. The results are seen in I, Fig. 3. The RTs of lentiviruses and HTLV-I showed a considerably higher activity in the Lenti than in the C-type assay, while the opposite was the case for FeLV RT and the activity found in the murine cell line SP2/0. For the remaining part of the study, this activity was treated as an “unknown” sample, as an example of the use of the system for identification of RTs.

To further highlight the concept of RT identification, we performed a study on the relationships between RTb-Ab against MMuLV and a few other viruses. In this part of the work we used sera with high titers of RTb-Ab against MMuLV RT, obtained from mice immunised with recombinant MMuLV RT. The latter was originally done as part of a never finalized project to produce MAbs against MMuLV RT.

The reactions between the anti-MMuLV RT sera and selected RTs are shown in I, Fig. 4. The sera gave strong blocking of MMuLV, FeLV and SP2/0 RT, while no blocking was obtained of HIV-1 or FIV RT. The data indicated that the SP2/0 activity originates from a virus related to MMuLV.

To determine the specificity of the assay, we tested several supernatants from cell cultures of different origin, as well as sera from humans, mice and cats. None of the tested materials gave any activity in the Lenti assay, while two of the supernatants showed activity in the C-type assay. These were from propagation of the mentioned SP2/0 cells and the pig cell line PK-15, which is known to express PERV [190]. It should be noted that in this study human serum but not plasma was tested for RT activity. Later work has shown that human plasma contains RT like activity, which is further discussed in connection with III.

To summarize, the data in this study show that the C-type RT assay is useful as a tool for discrimination between γ-retroviruses and particularly lentiviruses, both in direct RT assay and by determination of isozyme specific RTb-Abs.

RT vs. p24 for quantitation of HIV-1 (II)

This paper illustrates the usefulness of RT as a marker for quantitation of HIV-1. Here, we used the Lenti-RT assay for quantitation of HIV-1 of different subtypes from cell culture, in comparison with data from two p24 Ag assays. The study was initiated due to the fact that at the time, the knowledge was poor about the ability of p24 assay to detect different HIV-1 subtypes.
Two panels of HIV-1 isolates were studied, each consisting of ten isolates: Panel A, isolates of subtype B, all originating from Italy; panel B, isolates of different subtypes and origins. All were grown at identical conditions in the lab of our collaborators.

The RT activities of all samples were determined with the Lenti RT kit, and the p24 antigen by the use of two assays. These were one in house ELISA used by our collaborators [260] and a commercially available HIV-1 p24 Ag kit from Abbott. Both were based on anti-p24 Abs directed against HIV-1 of subtype B.

The ratio between the amount of p24 and the RT activity was calculated for each isolate (II, Tables 1 and 2, Fig. 1). Most of the isolates gave ratios in a similar range, but a few from panel B gave very low ratios. These were the two tested group O isolates, and one isolate each of subtype A, B and C. The reason why this subtype B isolate was underestimated by p24 is unclear.

Additionally, there were differences between the results obtained with the two p24 assays, indicating that the anti-p24 Abs in the in-house and Abbott kits had different specificities. In summary, the data indicate that RT activity as a marker of HIV infection is less dependent on genetic variations than p24, a fact suggesting the possibility to use RT from patients for VL determinations.

The ExaVir Load method

Development of version 1 (III)

Basic considerations and overview

The idea behind ExaVir Load version 1 (EVLv1) was to utilize our knowledge of RT assays for measuring a clinically important parameter of HIV infection. The possibility to use RT as a marker for VL had been implied by the results presented in II, as well as by other studies [34, 78]. The improved sensitivity obtained in the HS-Lenti assay (Table 5) suggested a possibility to measure RT amounts small enough for determination of VL.

However, we had previously observed that human plasma contains disturbing factors that make direct determination of small amounts of HIV RT very difficult. The most important of these factors is RTb-Ab. Only samples with high VL, mainly taken during primary infection, were possible to measure in direct RT assay [11]. Therefore, we needed to develop a procedure for separation of the virions from plasma, to be carried out before determination of RT activity.

An overview of the developed separation procedure is shown in Fig. 5, and the separation equipment is seen in Fig. 6.
First, a solution of the protein reactive agent DTNB is added to a tube (1.). The plasma sample, 1 ml, is then added (2.-3.) and the tubes incubated for 1 h at room temperature (4.). After this, 1.5 ml of a gel slurry is added to each tube (5.), followed by another incubation at room temperature, now on a shaker at low speed, for 90 min (6.). During this incubation, the virus particles present in the plasma are immobilized on the gel. The sample/gel mixture is vortexed (7.) and poured into a plastic column mounted into a washing device (8.). The supernatant is then removed with the help of vacuum from a pump (9.). After this, the gels are washed four times with a wash buffer (10.-11.) and twice with a reconditioning buffer (12.-13.). Finally, a detergent containing lysis buffer is added to the gel (14.), and the purified RT (lysate) is eluted by vacuum (15.) into a plastic tube. The RT activity of the lysate is finally determined. This activity is proportional to the amount of HIV virus present in the original plasma sample.

At the time when the separation procedure was developed, our group had very limited access to HIV material. Therefore we used culture derived FIV as model virus during the development of the basic method. The use of FIV
also simplified the work because of the lesser need for security facilities. The results obtained with FIV were later confirmed to be valid also for HIV-1.

Beside the publications included in this thesis, the method has so far been documented in two patent applications [132, 149] as well as in more clinical studies [31] [Greengrass et al, submitted, Seyoum et al, manuscript], and in several conference presentations.

The pretreatment step

The new VL technique was from the beginning intended for use with the same type of patient plasma as is tested with the RNA based methods. We knew from before that human plasma (but not serum, see I) displays RT-like activity, that could be detected in our assay systems. During the development of the test (see following sections), we found that varying amounts of this activity could be recoverd in the system, depending on the separation conditions used. Generally, the use of low pH and high ionic strength in the binding and washing steps gave a recovery of RT-like activity of 1% or less of that in the plasma (III, Table II). However, we recognized a significant risk that false positive results still could occur, especially since the nature of the false activity was not known and variations between individuals could be expected.

Therefore we evaluated the ability of several substances to inhibit the false positive plasma activity without affecting the activity of HIV-1 RT. The substances tested were various kinds of protein modifying reagents, some of which were known to modify thiol groups in proteins, e.g. in cysteine residues. One of the best substances in this respect was DTNB (Ellman's reagent), which is normally used for analysis of the amount of free thiols in a protein sample [215]. With DTNB added directly to the RT assay at 50 µM, the RT-like activity of plasma was almost eradicated while the activity of recombinant HIV-1 RT was not altered (III, Fig. 3). However, the RT mutant Y181C displayed a significantly increased sensitivity to the additive. This was no surprise, as wildtype HIV-1 RT has two cysteine residues, and this mutant carries one extra that could be modified by DTNB [107, 144]. It was thus not feasible to achieve an RT specific assay by addition of DTNB to the reaction solution. Therefore we attempted to add DTNB as a pretreatment to the plasma sample prior to the binding step. This showed to have the desired effect, as no RT like activity was recovered. The high final concentration used, 20 mM in the plasma sample, was chosen simply to minimize the risk of remaining RT-like activity. This concentration was shown not to affect the recovery of HIV-1 RT, and the DTNB possibly recovered at lysis was not able to disturb the activity of RT with the Y181C mutation (III, Table III). Also, our model virus FIV was not susceptible to this sample treatment.

The exact origin of the RT-like activity in human plasma has not been elucidated. However, the few experiments performed suggest that the activity
is contained within some kind of structure, as it is possible to pellet by high speed centrifugation. The RT assay preferences of the activity is such that we suspect it to be mitochondrial $\gamma$-polymerase. This enzyme is, however, more exposed to modification by DTNB than the HIV RT, which is protected by the viral membrane. As the pretreatment obviously has solved the problem for the VL assay, there are currently no plans for further work to address the question of the origin of this activity.

The solid matrix and the binding step

For the choice of solid matrix, 82 different commercially available gels were tested for their ability to bind FIV that had been added to blood donor plasma. All gels were tested for their ability to remove FIV (measured by RT activity) from the added sample, and the recovery of added FIV RT from the gel was measured as well. The best results were obtained for anionic ion exchange media, mainly with DEAE or TMAE as active group (III, Table I). After some time of combined testing of a few gels for the binding conditions, the Fractogel EMD TMAE Hicap, from Merck, was chosen for further separation development. This type of gel is known to bind human IgG poorly at a pH below 6 [255], a fact that was promising for the further work.

The conditions during the binding of the virus to the gel was optimized empirically. Our goal was to bind as much virus, and as little of potentially disturbing factors, as possible. Particularly, we would like to avoid to bind Ig, as the presence on the gel of Abs binding RT could possibly prevent the enzyme from being recovered. Also, RTb-Ab recovered from the gel could block the enzyme activity in the assay. Here we had good help of an in-house ELISA for human Ig, by which we could estimate the recovery of antibodies from the sample. It was soon apparent that use of a high concentration of chaotropic ions and a low pH reduced the recovery of Ig but allowed a good binding of FIV. The best results were obtained with approx. 250 mM of KI at a final pH of 5.4 (III, Fig. 1). The buffer substance MES, chosen because of a suitable buffering range, was used at a concentration of approx. 200 mM, in order to counteract the buffering capacity of the plasma.

The wash and reconditioning steps

The purpose of the gel wash was to remove as much as possible of Abs or other potentially disturbing factors without removing any immobilized virus. As for the binding step, the best removal of Ig was obtained at high salt concentration and low pH. However, the final recovery of RT was affected by the presence of KI or KCl in the wash buffer. Therefore 500 mM KAc was used (III, Fig. 1). As buffer substance we still used MES, but only at 10 mM as no competing buffer was present.

The RT activity assay is performed at low salt concentration at pH 7.6. The conditions of the wash buffer is thus far from optimal for the RT enzyme, and we wanted to avoid the inclusion of wash buffer in the assay. There-
fore a reconditioning step was introduced. The reconditioning buffer is similar to the reaction buffer of the RT assay, but does not include primer, substrate or detergent.

The washing device used was originally a commercially available vacuum manifold from Supelco, made for 24 columns. After the work described in III was finished, a device for 32 columns was custom designed for the separation procedure by a local technical company, after ideas from Cavidi Tech. This device is more convenient for the system, as it does not include any tubings or other loose parts that need cleaning or could mean risks for contaminations. In IV, this new equipment was used. The current washing device and other separation equipment is shown in Fig. 6.

Figure 6. The ExaVir separation equipment. A: Tubes for pretreatment/gel binding in box (lid to the left); B: Upper part of washing device (column holder), with columns; C: Lower part of washing device (waste collector), with tube to pump; D: Pump, connected to waste container (outside picture); E: Buffer dispenser for wash and reconditioning; F: Lower part of washing device for lysate collection (sample collector); G: Lysate tube rack with tubes. Photo: Staffan Sjödahl

The lysis step

The RT in the virions immobilized to the gel should finally be eluted for activity measurement. As the reconditioning buffer already had created a suitable environment for the RT, the lysis buffer was mostly the same, with detergent added. At first, we attempted to use the detergent Triton X-100, as this is included in the RT assay. However, when the vacuum was applied, Triton X-100 gave rise to a very large amount of foam. This could potentially be a cause of contamination between the columns or collection tubes, and we therefore started a search for an alternative detergent. After testing several, some of which did not cause foam but disturbed the RT, we finally deci-
ded to use Brij 30 (polyethoxylate 4-lauryl ether). This detergent gave considerably less foam than Triton X-100 but a similar RT recovery.

The RT assay
The HS-Lenti assay was used as basis for the RT measurement. However, to increase the sensitivity we attempted to use a larger volume of sample in the assay (75 instead of 50 μl). This was successful, and the RT assay was used without any further modifications, except the alterations of the conditions resulting from differences between the lysate and the original base buffer. To obtain a better range of detection, also a five-fold dilution of the lysate was assayed (15 μl). We knew from earlier studies that the activity of HIV-1 RT is linear with time for up to 48 h. Therefore, an over night incubation was used, typically for 17 h, to obtain a good assay sensitivity. As the so far performed steps of the system with the current protocol takes about four hours, we did not see any need to attempt the use of a shorter assay time.

In the study described in III, both colorimetric and fluorimetric detection of the RT assay product was used. The fluorimetric detection gave a somewhat better sensitivity, with a shorter AP reaction time. With colorimetry, an over night incubation needed to be performed to obtain a sensitivity close to that with fluorimetry.

The RT activity obtained for each sample was recalculated into fg RT/ml plasma, with the help of a serially diluted HIV-1 RT standard included on each plate. The calculations made were based on assumptions about RT recovery and relationship between plasma and lysis buffer volumes, giving rise to a constant value by which the RT activity of the lysate was multiplied. Concerning the cut off for positivity in the RT assay, in III a criterion was used corresponding to at least two times the average assay background.

Results obtained with version 1 (III)
RT purification
The purification of the RT from disturbing factors was the most important task to accomplish with the separation procedure. In some samples, this purification was not complete, as the relationship between the activity found in 75 and 15 μl of lysate was not five fold. On average, there was an approximately four fold difference between the activity of the two lysate volumes (data not shown in III). Because of these occasional disturbances, for samples giving a clearly higher fg/ml value for 15 μl of lysate the value from the smaller volume was used. This should in most cases not be a problem, with the exception for samples with very low VL for which only 75 μl of lysate gave a positive signal.

The reasons for these remaining disturbances are not clear. The problem could potentially be due to residual RTb-Ab, drugs or other, currently unknown, factors. Concerning the possibility of residual RTb-Ab to cause
disturbances, additional (unpublished) experiments, using samples from HIV infected persons with undetectable VL by RNA, suggest that RTb-Ab are not recovered in sufficient amounts to disturb HIV-1 RT added in assay.

In III, Table III, results concerning several other possible interferences are shown. Of the RT directed anti-HIV drugs tested, only EFV was recovered in sufficient amounts to cause inhibition of HIV-1 RT in assay. However, it should be noted that in these spiked samples, the drugs had not passed the metabolism of the patient and thus are suspected not to be attached to proteins in the way they clinically are [27]. Also, interferences due to EFV could not be seen in the clinical data, as the RT/RNA relationship for EFV containing samples was similar to that for samples from treatment naive patients (See Clinical considerations). This is supported by another study [Greengrass et al, submitted] suggesting that EFV treatment does not interfere with the RT determinations, in spite of the tight binding of EFV to the RT enzyme [168]. Problems due to residual DTNB in the lysate are unlikely, as no effect on the susceptible RT mutant was observed (III, Table III).

RT load determinations

Viral load measured by RT was determined for a total of 390 samples from Swedish patients. As some of them were represented by more than one sample, to avoid bias the last sample taken from each patient was chosen for further calculations. The overall correlation between RT load and VL according to Roche Amplicor 1.5 ultra sensitive (III, Fig. 4) was strong (r=0.90), and with fluorimetry, all samples with >6 900 RNA copies/ml had detectable RT (III, Fig. 4 and Table VI). With colorimetry, four samples with >6 900 RNA copies/ml were RT negative; the highest RNA value among these was 30 400 copies/ml.

Since the RT/RNA ratios varied considerably in spite of the strong correlation, a definite detection limit for RT load, expressed as RNA copies/ml, could not be given. However, between 2 500 and 6 900 copies/ml, 50% of the samples were RT positive (III, Table VI). The detection limit for fluorimetry could thus be found somewhere in that range. The results in IV suggest that the detection limit of EVLv2 is around 400 copies/ml, and indicates that the difference between the versions is about 10 fold. This gives an approximate detection limit of EVLv1 at 4 000 copies/ml, a value consistent with the data presented in III.

The Swedish patient cohort is supposed to consist mostly of patients infected with subtype B. A panel of HIV virus of ten different subtypes were tested for RT load, and all of the samples were detectable. A parallel study [31] did also show the ability of the method to quantitate different subtypes, as well as HIV-2. The usefulness for all types of HIV is no surprise, since the binding of virus to the gel should be dependent on general chemical features of the virus membrane (as e.g. FIV also binds) and the RT activity measured is generally conserved.
Many of the clinical samples tested in III (143) were undetectable by RNA, and of these only one sample gave a low RT load. A number of plasma from Swedish blood donors were also tested, in order to find out the specificity of the test. Of the 202 samples tested, 198 were RT negative and the remaining four gave low values, no doubt due to random background variations. The background signals of the experiments on clinical samples included in III had a considerable variation, larger than later obtained, in our and other labs, using EVLv1.

Clinical considerations

As there were more than one sample from 69 patients in our material, we made an attempt to compare the development in VL determined by the two methods over time for each patient. The results, shown in III, Table VII, show that the amount of RT/RNA ratio is relatively constant over time for each patient, and that the RNA copy number and RT activity in most cases covariate.

More clear results on this issue have been obtained in other studies [31] [Greengrass et al, submitted, Seyoum et al, manuscript] where longer series of consecutive samples from patients were tested. The results indicate that the RT load in general follows the VL according to RNA. All these studies, including III, also show that although relatively constant for a given patient, the relationship between RT and RNA amounts vary considerably between individuals. This fact has previously been observed by others [78] and requires further investigation, as it could potentially include clinically relevant information.

Many of the clinical samples tested in III were from patients on treatment. As could be expected, only very few of these showed detectable RT activity. A larger part of the sample material was from patients on pause of treatment. When the relationships between fg RT/ml and RNA copies/ml were analysed, all these groups (naive, pause and treatment) generated almost identical linear regression lines (III, Fig. 4). This suggests that current or previous presence of treatment in the patient does not alter the relationship between the amount of measurable RT and RNA, which further underlines the relevance of using RT as a VL marker.

In III, it was not possible to see any correlation between VL and CD4 cell number. One possible reason for this is the fact that in Sweden a patient with declining CD4 cell numbers will be treated. A recent study using EVLv1 on plasma from Ethiopian treatment naive patients [Seyoum et al, manuscript] suggests that VL has a significant inverse correlation to CD4 cell number, and to future changes in this parameter. However, the correlation appears to be better for VL measured by RT than by RNA, indicating that RT could be a more reliable marker than RNA for infectious virus particles.
Version 2 (IV)

Differences to version 1
EVLv1 was the first official version of the HIV RT load test presented to the scientific community. It had, however, been preceded by several prototype versions, and a prototype of EVLv2 was already existing by the time III was published. The reason for this was observed limitations of EVLv1. Particularly, the sensitivity achieved was not as good as we initially had hoped. Although we had noted the presence of disturbances in some samples, we found that there could be a possibility to increase the sensitivity by using a larger amount of lysate in the RT assay. With a more concentrated reaction buffer, we had the idea to put 150 μl of lysate in each well. Also, another idea was to prolong the RT assay time, but also in this case we recognized a risk for greater problems, with the disturbances affecting the assay linearity with time. In the final EVLv2, 150 and 30 μl of lysate and 41 h of RT reaction was used. The disturbances, seen as a difference in activity between the sample amounts of less than five fold, were not more prominent for the final EVLv2 than for EVLv1.

In an attempt to address the issue of disturbances, we tried different additions to the gel wash buffer. Most of these were detergents and similar compounds we had reason to believe would not destroy the virions on the gel.

One of these substances had a positive effect, namely Triton CF-32, a low foam surfactant normally used for industrial cleaning purposes. The effect of 0.3% Triton CF-32 in the wash buffer was an increased RT recovery for many samples. On average, 16% more RT was recovered, although the gain was up to 50% for some samples. The introduction of the addition did not give rise to any changes in the relations between 150 and 30 μl of lysate.

We also made further optimizations of the lysis buffer. Several alterations were made, of which the most important were the increase in Mg\(^{2+}\) concentration and the change of detergent from Brij 30 to Brij 56. The new lysis buffer resulted in an equal RT recovery, but also seemed to increase the velocity of RT in the assay as well as the time of a linear RT reaction (Table 5; IV, Table I).

The work with EVLv2 was greatly simplified by the access to HIV-1 positive plasma material, bought from the South African National Blood Service in Johannesburg.

Improved performance
The overall difference in detection sensitivity between EVLv1 and 2 was approximately ten fold, and the mean difference for a panel of HIV-1 positive samples was even larger. The improvements were due to increased signals obtained in the RT assay, but also due to lower backgrounds using EVLv2 (IV, Table I).
In this table is also seen that the use of 17 h of RT assay instead of 41 h still gives an increase in sensitivity of about six fold. As the detection limit using 41 h assay is around 400 copies/ml, this would principally mean that if a detection limit of 1000 copies/ml is good enough, an over night assay time would be sufficient. However, as the linearity of the RT with time differs between samples, different assay times on different occasions should be avoided in order to get comparable results. So far, the laboratories using EVLv2 are instructed to keep the RT assay time at 36 to 48 h.

To determine the clinical usefulness of EVLv2, a similar study of correlation to Roche Amplicor as in III was performed (IV, Fig. 2). The samples were from the same patient cohort and collected during the same period of time as in the previous study. The overall correlation between RT by EVLv2 and RNA was also the same as for EVLv1 (r=0.90). The amount of RT positive samples with low VL according to RNA was greatly increased compared to in III. Now, 90% of all samples with >400 HIV-1 RNA copies/ml gave detectable RT activity (IV, Table II), and in the interval 400-1 500 copies/ml, 71% of the samples were RT positive with colorimetry. This should be compared to III (Table VI), where no sample in the interval 1 200-2 500 copies/ml was detectable. The figures highlight the greatly improved sensitivity of EVLv2. The fact that a small percentage of samples with larger VL according to PCR still were RT negative should be regarded as a result of the two methods measuring different viral parameters. Possibly, virus degradation during storage could contribute.

In IV, all patient samples with undetectable RNA also had undetectable RT. One of 58 HIV negative blood donors had a low RT load, but only when using colorimetric detection, a fact indicating that the reason was background variation.

The results in IV indicate that EVLv2 could be a useful alternative to RNA determinations for VL measurements. The ability of the method to detect different types of HIV, together with its relative simplicity, indicates the usefulness of the method in resource limited countries. For reasons mentioned in III and IV, as well as in the Introduction, RT activity may have advantages for VL determinations, making ExaVir Load interesting also for use in the developed world.

Purified enzyme used for drug susceptibility testing (V)

With RT purified from the virus particles of a patient available, the possibility for direct characterization of the RT arises. The characteristics that are most interesting to find out about an HIV patient under treatment is whether the virus of the patient has become resistant to any of the drugs included in the current treatment. Also, when treatment is going to start, it is of value to determine if the patient is infected with virus already resistant to any of the drugs in the planned regimen. Therefore the ExaVir Drug method, of which
a prototype is described in V, was developed. Besides in V, the method has been documented in a patent application [243].

The principle of the DS determination using ExaVir Drug is simple. The RT purified by the ExaVir separation is assayed together with a serial dilution of the drug. From the inhibition obtained from the different drug concentrations, a profile of susceptibility of the RT is obtained, and an IC₅₀ value is calculated. By comparison of the profile with those of wildtype and resistant standard RT, the level of resistance in the sample can be assessed.

Such profiles are illustrated in Fig. 7. In the figure is seen typical results obtained for a strongly resistant, a moderately resistant and a wildtype sample, when testing for resistance to AZT-TP. The obtained IC₅₀ values are marked on the x-axis.

![Figure 7. Illustration of typical susceptibility profiles for AZT-TP.](image)

Two categories of drugs are possible to use in the current DS system. These are NNRTIs, and NRTIs that are analogues to thymidine (AZT and d4T). The NNRTIs act directly on the RT enzyme and the nature of the template is not involved in their action. The NRTIs, on the other hand, act as terminators of the growing DNA strand. Therefore only NRTIs that are complementary to the RNA template could be used in the assay.

For the NNRTIs, the standard RT assay conditions described in III could be applied. For the NRTIs, however, several alterations to the reaction conditions were needed to make DS determinations possible. As the excision reaction of resistant isolates is ATP dependent, this was added to the assay at an appropriate concentration (6 mM). Also, the BrdUTP concentration was
lowered in order to obtain a better competition between the assay substrate and the NRTI. Still, a poor resolution between susceptible and resistant genotypes was obtained when using the other conditions according to standard assay. Experiments showed that lowering the pH in the assay resulted in an improved resolution. The final pH of 7.0 was a compromise between resolution and activity, as the optimal pH for HIV-1 RT activity is 7.6 and the activity at pH below 7 was too low to be useful. Thus, a higher concentration of RT was needed for a lysate to be useful for determination of resistance to NRTIs than NNRTIs (200 and 70 fg RT/ml, respectively).

The method was first evaluated using recombinant RT with different resistance mutations (V, Table 1), and generally found to give the expected results. Then, the assay was tested on clinical samples in two panels. One panel consisted of samples with mutations connected with resistance to NNRTIs, the other with NRTIs. Both panels also included wildtype samples, and all samples were selected to have >10 000 RNA copies/ml in order to be possible to test.

In the NNRTI panel, 15 of 17 samples had enough RT to allow testing, in the NRTI panel 15 of 28. Some samples were possible to test in both systems. Of the samples possible to test, 10 samples had mutations giving rise to resistance against at least two of the NNRTIs (V, Table 3), and they also gave the expected resistance profiles. With the exception of three test results, all of these samples had high level resistance to all three drugs, which was consistent with the presence of multi NNRTI resistance mutations.

Eight samples had mutations connected with NRTI resistance (V, Table 4), and all of these also showed decreased susceptibility towards AZT and d4T (except two which were not tested for d4T).

The results in V, shown as resolution between wildtype and resistant patient samples, is illustrated in Fig. 8. The figure clearly shows that the resolution obtained was very good for the NNRTIs, while the NRTIs gave a more narrow pattern. There was no overlap between wildtype and resistant samples, but the close position in the figure illustrates that the assay data are not as easily interpreted for NRTIs as for NNRTIs.

The data in V indicate that DS testing is a potentially important application of the EVL method. This has lately been confirmed in an external study [273]. With the increased sensitivity obtained in IV also samples with lower VL are possible to test.
Use of the ExaVir concept for other retroviruses

A limited amount of work has been carried out to determine the usefulness of the separation technique for non-lenti retroviruses. Most work has been performed on PERV in supernatants from PK-15 and infected human 293 cells. The results show that PERV is extremely sensitive to iodide ions, giving no recovery when the standard binding conditions for HIV were used. However, with the KI replaced by KCl the binding was effective. Also, the virus is sensitive to DTNB. A considerably lower concentration (1 mM) could however be used without significant loss of activity. A low concentration of cysteamine was used in the lysis buffer to counteract recovered DTNB. For optimal performance, the KAc concentration in the wash buffer was also lowered to 0.25 M. The reconditioning buffer in the tested PERV system was adapted to the assay for γ-retroviruses, and the final assay gave a good recovery of PERV RT (>50%), when the virus was added to human plasma. At direct purification from culture supernatant the recovery was even higher. It was also found that tested blood donor plasma did not give any false positive signals at these conditions.

This prototype version of the system was used for determination of the sensitivity to human serum complement. PERV derived from the original PK-15 cell line and grown in 293 cells were mixed with fresh human serum.
After a short incubation at 37°C the samples were assayed for VL of PERV. The virus grown in human cells showed a good recovery of RT, while virtually no RT was recovered from the PK-15 derived PERV. These data, briefly described in [229], indicate that the 293 derived PERV can escape an important part of the human immune system. This confirms findings in [190], and may have implications for the possibility of future xenotransplantations.

Experiments on the RTs of several other viruses show that the sensitivity to KI and DTNB vary considerably. As mentioned earlier, the lentiviruses seem to be very insensitive, while all other tested RTs are more or less susceptible to one or both of these compounds. Among the tested viruses are MuLV, JSRV and BLV. This shows that the presented system for virus separation needs to be optimized for any individual type of virus, in order to be useful for further research.

Relations between the RT assays for HIV-1

Four different RT assays for HIV-1 RT have been involved in this thesis. These are the Lenti-RT ([60], I, II), and HS-Lenti assays [131, 148] and the RT assay parts of ExaVir Load version 1 (III) and 2 (IV). An experiment was performed to compare these kits, in terms of sensitivity, depending on assay time and mode of data evaluation. The normal assay times for EVLv1 and 2, 17 and 41 h, were used. The results are summarized in Table 5.

Table 5. Amount of RT (fg / ml) required to generate a signal corresponding to a detection limit of 2 *BG or (2 * BG) + (2 * stdev) according to the standard curve. Colorimetric detection, over night AP reaction. In parenthesis, the fold decrease in detection limit in relation to Lenti assay. Bold italics = assay time and mode of evaluation according to the protocol of the assay.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Time (h)</th>
<th>2 * BG</th>
<th>(2 * BG) + (2 * stdev)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No zero read</td>
<td>Zero read</td>
</tr>
<tr>
<td>Lenti</td>
<td>17</td>
<td>217 (1)</td>
<td>132 (1.6)</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>115 (1.9)</td>
<td>70 (3.1)</td>
</tr>
<tr>
<td>HS-Lenti</td>
<td>17</td>
<td>22 (9.8)</td>
<td>4.1 (53)</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>12 (18)</td>
<td>2.0 (109)</td>
</tr>
<tr>
<td>EVLv1</td>
<td>17</td>
<td>17 (13)</td>
<td>2.3 (96)</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>9.0 (24)</td>
<td>1.3 (165)</td>
</tr>
<tr>
<td>EVLv2</td>
<td>17</td>
<td>7.3 (30)</td>
<td>0.99 (220)</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>3.5 (62)</td>
<td>0.49 (444)</td>
</tr>
</tbody>
</table>
The data show that EVLv2 was 241 fold more sensitive than the Lenti RT assay, evaluated according to the respective protocol of the assays. Also, EVLv2 was here 4.4 fold more sensitive than EVLv1, which is clearly less than the 10 fold observed in IV, Table I. The reason for this is that in the experiment shown in Table 5, the assay backgrounds of EVLv2 were higher than in IV. Table 5 is, however, based on one single experiment while in IV, data from several experiments indicate a normal difference in background between the assay versions giving a larger difference in detection limit. The shown data indicate the very strong impact on the assay sensitivity by the use of the “zero reading”. When the background is defined as the increase in absorbance of the background wells, rather than the absolute absorbance values, it leads to a much lower cut off, as long as this is related to the assay backgrounds. The relation between the HS-Mn and C-type assays (data not shown) is similar to between HS-Lenti and Lenti (10 fold).
The present thesis illustrates some aspects of the usefulness of enzymatic RT activity assays for clinical and research purposes. The assay developed for MMuLV RT was shown not only to be useful for determination of \( \gamma \)-retrovirus RT, but also as a tool to distinguish between different RT isozymes. The ability of RT assay to quantitate different HIV-1 subtypes equally well indicated that RT activity is valid as marker for quantitation of HIV. In the case of VL, the presented work shows that data from EVL correlates strongly to an established RNA based method, and that EVLv2 also has a sensitivity comparable to this. The described prototype of ExaVir Drug offers a potentially important tool for determination of patient virus susceptibility for current or planned anti-HIV treatment. Due to their technical simplicity, and ability to detect a broad range of HIV-1 subtypes, the ExaVir methods could be interesting alternatives to the established VL and DS methods, particularly for use in resource limited settings.

There are several possibilities for further development of the ExaVir methods. An automation of the separation procedure would be most interesting for the user, but is otherwise outside the subject of this thesis. The knowledge about EVL needs to be complemented with studies of interferences from various potentially disturbing factors, e.g. whether infections from other viruses or other agents could influence the VL results. Also, the observed but not identified interferences in some samples, resulting in non-linear relationship between assayed lysate amounts, need to be addressed. A way to get rid of these disturbances could mean further increase in assay sensitivity. Experiments to learn more about this are currently underway.

Further increase in sensitivity of EVL would be valuable in several ways. First, samples with lower VL could be measured, also for DS. Second, the current RT assay time could be shortened, which would be beneficial for practical use and increase the test throughput. Third, the proposed use of EVL for HIV diagnosis at early stage of infection, before seroconversion, would be more realistic. This application could be useful not least for safety control of blood donors or donated blood bags.

Several ways to achieve better sensitivity have been proposed. On the RT assay level, these include the increase of the amount of detectable DNA product by the use of specially designed template/primer constructs, or by the introduction of a signal amplification system. Other ideas include concentration of virus from a larger volume of plasma by centrifugation. This has been tried and found to work in principle. A problem is to resuspend the virus pellet in a reproducible way before testing for VL.
Clinically, the variable relationship between RT activity and RNA copy number observed by us and others could possibly indicate important differences between virus populations. To address this issue would however require access to very special panels of patient material. The idea that the presence of more RT in relation to RNA could correspond to worse prognosis is so far only a tempting speculation.

ExaVir Load, as it is, is useful for other lentiviruses than HIV. A study on SIV in macaques, in connection to vaccine development, is currently being carried out, and the results so far are very promising (G Corrigan, personal communication). The obvious use for FIV in cats has so far not been tried.

The version of ExaVir Drug described here is limited for use with NNRTIs and T-analogues. A prototype assay for measurement of all NRTIs, based on a variable DNA template, has been developed but has so far a too low sensitivity for clinical use. Experiments with different template/primer constructs to create a more general DS test are underway. The DNA template plate has also been used for prototype assays for DNA dependent DNA polymerases. Possible use of these include measurements of DNA polymerases as markers for malignancies.

The lysates obtained from the VL procedure may also contain other virus components than RT. If so, determination of p24 or RNA could be performed and compared to other results. Also PR or IN could potentially be determined, provided the existence of appropriate enzyme activity assays.

As described in this thesis, the ExaVir concept has been developed and applied for lentiviruses. The possible use for other types of retroviruses is also very interesting. Beside the use for VL measurements of other retrovirus infections, the purification of intact virus particles suggests other applications on virions in culture supernatants or patient material, with the mentioned application for PERV as an example. Other research possibilities include identification of previously not known retroviruses. For this, the presence of RTb-Ab in an infected animal could be used as complement to indicate a virus as exogenous. RT extracted by the ExaVir separation could be characterized in different respects, such as assay requirements or drug susceptibility. Of course, improvements of RT assay in terms of sensitivity could be crucial for any new application of this kind. The combination of the current separation system with other techniques could in this context also be of interest. For instance, assay of lysate from EVL by extremely sensitive PCR based RT assay (Amp-RT or PERT) could potentially offer chances to locate minute amount of virion associated RTs, particularly with better optimized as well as isozyme specific conditions in the RT step. This could lead to unique research opportunities, such as search for sparsely expressed endogenous retroviruses. Finally, the separation technique may be possible to adapt to other virus types than retroviruses, provided the outer envelope of the virus has the ability to bind to a gel.
Retrovirus är en stor grupp virus, vars mest kända representant är HIV (mänskligt immunbristvirus). Retrovirus finns hos snart sagt alla djur, och orsaker sjukdomar hos många. Ett av de viktigaste enzymerna hos alla retrovirus är omvänt transkriptas, RT. Det ombesörjer att virusets arvsmassa, som består av RNA, översätts till DNA, som blir en del av cellens arvsmassa när viruset infekterar en cell. Denna avhandling handlar om utvecklingen och användningen av metoder för mätning av aktiviteten hos RT.

Ett av de mest studerade retrovirusen är MMuLV, Moloneys musleukemivirus. Sedan upptäckten på 60-talet har detta virus kommit till stor användning som modellvirus för studium av retrovirus i allmänhet och för olika forskningsändamål. MMuLV tillhör en grupp som kallas gammaretrovirus, som finns hos många djur men inte hos människor.

Hos människa finns dock fyra kända smittsamma retrovirus. Två av dessa är HIV typ 1 och 2. HIV tillhör en grupp som kallas lentivirus, och orsakar som bekant sjukdomen AIDS (förvärvat immunbristsyndrom). Den uppstår genom att HIV dödar viktiga celler i kroppens immunförsvar, varför detta gradvis försvinner tills det inte längre kan försvara kroppen mot bl a infektioner. Sedan HIV började spridas i början av 80-talet har ca 20 miljoner människor dött till följd av dessa händelser. I västvärlden har vi numera i stort sett fått bort detta problem, det är ändå katastrofalt i stora delar av tredje världen, särskilt i Afrika söder om Sahara.

Sedan ett antal år tillbaka finns fungerande s k bromsmediciner, som håller infektionen av HIV nere genom att hindra virusets förökning i kroppen. På så vis kan inte viruset döda så många celler och därmed tar det längre tid tills patienten utvecklar AIDS. Dock har viruset en förmåga att utveckla resistens, motståndskraft, mot dessa mediciner, så att de inte längre har avsedd verkan. Resistensens uppstår genom att virus som har vissa mutationer har lättare att föröka sig i kroppen än icke-resistent virus. När viruset blivit resistant märks detta genom att patienten får en kraftigt ökad mängd virus i kroppen. Det är därför viktigt att kunna mäta mängden virus i patientens blod, vilket kallas virusbörda (VL). VL måts oftast med moderna molekylärbiologiska metoder, som PCR (polymeras-kedjereaktion), genom vilka mängden virus-RNA hos patienten måts. När virusbörden stiger p g a resistens behöver behandlingen ändras till en som viruset inte är resistant emot, annars riskerar patientens tillstånd att kraftigt försvamas. För att kunna veta vilken ny behandling patienten skall ha, eller vilken som ska väljas när en patient skall börja behandlas, kan man testa huruvida patientens virus är känsligt för olika mediciner. Detta görs antingen på genotypnivå, genom att man söker efter de mutationer som orsakar resistens, eller på fenotypnivå,
genom att man undersöker om patientens virus kan växa i närvaro av medicinen ifråga. Båda metoderna är dyra, komplicerade och tar lång tid.

Arbetet i den här avhandlingen handlar alltså om metoder för att mäta aktiviteten hos RT. Mätningarna sker utgående från en grundmetod, som varieras beroende på från vilket virus RT kommer.

Som tidigare nämnts översätter RT virusets RNA till DNA. I vårt fall sitter ett stycke konstgjort RNA fast i botten på de 96 brunnarna i en plastplatta. I en tillsatt reaktionsblandning finns den också konstgjorda DNA-bygGSTen BrdUUTP. RT, som tillsätts i ett prov, bygger med RNAt som mall ett DNA av BrdUUTP, som sedan kan detekteras m h a antikroppar mot DNA kopplade till ett enzym (AP). Detta mäts slutligen genom att man tillsätter ett ämne som i närvaro av AP färgas gult. Graden av gul färgutveckling är proportionell mot mängden RT i provet.

Den första delen av avhandlingen handlar i första hand om RT från det nämnda viruset MMuLV. Detta RT har annorlunda krav på den kemiska miljön i reaktionsblandningen jämfört med HIV-RT. En reaktionsblandning för MMuLV-RT togs fram utgående från en redan befintlig för HIV-RT. Resultatet blev en känslig metod för mätning av MMuLV-RT, som visade sig användbar även för RT från besläktade virus, såsom katt-leukemivirus (FeLV). Den nya metodens förmåga att mäta RT från olika retrovirus jämfördes med den ursprungliga optimerad för HIV. Här visades hur de båda metod-versionerna kan användas för att identifiera ett RT vars ursprung inte är känt. Detta kan ske dels genom jämförande av de olika RT-enzymernas krav på reaktionskonditioner, dels via reaktion med enzymspécifika s k RT-blockerande antikroppar, vilka i regel finns hos ett smittat djur.

Den befintliga mätmetoden för HIV-RT användes i avhandlingens nästa del. Här jämfördes dess förmåga att mäta mängden HIV-1 i ett odlingsprov med en annan etablerad metod, nämligen mätning av virusproteinet p24. Försöken visade att RT gav en säkrare bestämning än p24 av mängden virus, särskilt när HIV-1 var av vissa s k subtyper. Dessa kan vara svåra att mäta med andra metoder, eftersom de genetiskt skiljer sig ganska kraftigt från varandra och därmed deras p24 är olika. Aktiviteten hos RT är dock oberoende av subtyp, vilket gör RT-mätning mer pålitlig för att bestämma mängden HIV.

En ny metod för att mäta VL hos HIV-patienter behandlas i nästa del av avhandlingen. VL mäts normalt i plasma, d v s blod från vilket blodcellerna tagits bort. Plasma innehåller störande faktorer, bl a RT-blockerande antikroppar, som gör direkta mätningar av RT-aktivitet svåra. Därför behövde vi utveckla ett reningssteg för att kunna mäta RT-aktivitet från virus i plasma.

Framrening av viruspartiklarna ur plasma sker genom att plasmaprovet skakas med ett slags pulver, en gel, varvid viruspartiklarna binder till gelen. Gelen förs sedan över till en liten kolonn av plast, i vilken den tvättas m h a en tvättvätska med lågt pH-värde och hög salthalt. Merparten av antikroppar
och andra störande faktorer avlägsnas genom denna procedur. De till gelen
bundna viruspartiklarna slås sönder med en speciell lösning, varefter RT-
aktiviteten i provet som kommer ur kolonnen bestäms. RT-aktiviteten är
proportionell mot VL i plasmaprovet, och metoden (ExaVir Load) visar god
överensstämmelse med en av de etablerade VL-metoderna (Roche Amplicor
PCR). Den andra av de två här presenterade versionerna av metoden har en
känslighet att mäta HIV som ligger i närheten av den som de etablerade me-
toderna har. Den nya VL-mätmetoden har flera fördelar mot de etablerade.
Den är tekniskt enkel att utföra, utrustningen är relativt enkel och billig och
metoden kan utföras i vanliga laboratorier. Den har också fördelen att den
can mäta alla sorters HIV lika väl, vilket är särskilt viktigt i t ex Afrika där
alla subtyper finns. Den främsta nackdelen är att metoden tar relativt lång
tid. Tanken är att metoden i första hand skall kunna användas i tredje värl-
den, där man har behov av enkel och billig men ändå säker teknik för att
följa patienters sjukdomsutveckling.

Slutligen har även en ny metod att mäta känsligheten mot bromsmedici-
nern hos patientens virus utvecklats, utgående från den nya VL-metoden.
Principen är att man mäter RT-aktiviteten hos provet från VL-proceduren i
närvaro av olika mängder av den medicin man vill undersöka känsligheten
mot. Genom att jämföra med medicins påverkan på resistent respektive
naturligt RT kan virusets grad av resistens bestämmas. Metoden är hittills
utvecklad för bestämning av resistens mot två typer av mediciner som riktar
sig mot RT-enzymet, varav den ena omfattar den välkända medicinen AZT.
Även denna metod bör kunna användas i tredje världen för att undersöka
resistensutveckling hos patienter. Bromsmedicinerna har länge varit för dyra
för att komma till stor användning i t ex Afrika, men nyligen har detta börjat
ändras så att behandling i större skala kunnat påbörjas även där, och då ökar
också behovet av att kunna mäta resistens mot medicinerna.

Den viktigaste slutsatsen av denna avhandling är att mätningar av RT-
aktivitet ser ut att vara ett bra sätt att få kliniskt viktiga data om HIV-
patienters tillstånd. De presenterade metoderna kan också fungera som nytti-
ga verktyg i olika forsknings-tillämpningar, t ex i kombination med olika
reaktionskonditioner, för att undersöka egenskaper hos retrovirus.
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Yesterday my history
dreams are still a mystery
This living is a gift I should know
Jon Anderson

66
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67


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