A molecular diagnostic tool for improved treatment success of Human Adenovirus infections.

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SUMMARY

Human Adenovirus (HAdV) comprises 51 known serotypes and the viral infections caused by these viruses are frequent. Before the age of 3, approximately 80% of all humans have been infected by HAdV that often causes colds and diarrhoeas. HAdV infections in immunocompetent people are usually harmless. However, in immunosuppressed patients there is a high mortality rate due to HAdV infections, especially in stem cell transplant (SCT) patients where there is up to 60% mortality. The current antiviral drugs against HAdV infections do not have a well proven efficiency. Early detection of infection improves the success of the antiviral drugs, leading to an increased survival rate for these patients.

The aim of this study was to develop a prototype for a diagnostic assay for determination of HAdV, including the most frequent serotypes HAdV-1, 2, 5, 11, 34 and 35, in immunosuppressed patients. I used a molecular diagnostic technique called real-time PCR for the detection of HAdV. Real-time PCR is a highly sensitive method that continuously measures the PCR-product throughout the PCR. The design of efficient primers is essential for a highly sensitive real-time PCR assay. When efficient primers are established a more custom-made fluorescent probe is required. In this study Scorpions were used. Scorpions are amplicon-specific probes which follow the principle that a fluorescent signal is generated only if the Scorpion hybridizes to its complementary target. Non-specific amplification due to mispairing or primer dimer does not generate a signal.

Since HAdV-2 was the only variant from which DNA was relatively easy to acquire, this evaluation focused on HAdV-1, 2 and 5 by using HAdV-2 DNA. HAdV-1, 2 and 5 are identical in the target region where the primers were designed and therefore the primers designed for HAdV-2 DNA could also be used for detection of HAdV-1 and HAdV-5 DNA. In addition, the other important serotypes HAdV-11, 34 and 35, could be detected by including base variations in the primers in positions where these HAdV strains diverged in the primers, allowing the designed primers to detect all serotypes common in immunosuppressed patients.

This study is a first step towards the development of a diagnostic test for HAdV in immunosuppressed patients. With an efficient and sensitive test, HAdV may be detected prior to clinical symptoms of infection, leading to a better and earlier diagnosis that hopefully will reduce the mortality caused by this virus in immunosuppressed patients.

Because certain places in the genome of HAdV are very similar among the serotypes this study confirms that it is possible to design primers efficient to recognise several of these serotypes. However, since the efficiency of the primers and scorpions designed in the study was insufficient more time is needed to improve the primer design before a prototype can be developed.
# TABLE OF CONTENTS

**INTRODUCTION**.................................................................................................................. 1

ADENOVIRUS .................................................................................................................... 1
CONVENTIONAL AND REAL-TIME PCR ................................................................. 1
CONVENTIONAL PCR ..................................................................................................... 2
REAL-TIME PCR ............................................................................................................ 2
NONSPECIFIC REAL-TIME PCR DETECTION ............................................................. 4
SPECIFIC REAL-TIME PCR DETECTION ...................................................................... 4
HOW DO SCORPIONS WORK? ....................................................................................... 4
DESIGN OF PRIMERS AND SCORPIONS .................................................................... 5
THE AIM OF THE PROJECT ............................................................................................. 6

**RESULTS**......................................................................................................................... 7

PRIMER DESIGN ................................................................................................................ 7
YOPRO EVALUATION OF DESIGNED PRIMERS ....................................................... 8
STANDARD CURVE OF DESIGNED PRIMERS .............................................................. 9
IMPROVEMENTS OF AMPLIFICATION EFFICIENCY AND SENSITIVITY OF THE PCR 9
SCORPION DESIGN .......................................................................................................... 10
SCORPION EVALUATION ............................................................................................... 12
EVALUATION WITH POSITIVE CONTROL ................................................................. 14
DNA SEQUENCING .......................................................................................................... 15

**DISCUSSION**.................................................................................................................. 16

DNA SEQUENCING ......................................................................................................... 16
HYPOTHESIS FOR THE LOW EFFICIENCY .................................................................... 16
CONCLUSION .................................................................................................................... 17

**MATERIALS AND METHODS**....................................................................................... 18

VIRAL DNA MATERIAL .................................................................................................... 18
PRIMER DESIGN ............................................................................................................... 18
EVALUATION OF PRIMER WITH YOPRO .................................................................... 19
SCORPION DESIGN .......................................................................................................... 19
GENERATION OF POSITIVE CONTROL ....................................................................... 19
SEQUENCING OF VIRAL DNA ........................................................................................ 19

**ACKNOWLEDGEMENTS**............................................................................................... 21

**REFERENCES**................................................................................................................. 22
INTRODUCTION

Adenovirus

Human Adenoviruses (HAdVs) are nonenveloped, double-stranded DNA viruses that can be divided into six characterized major subtypes (A-F) and 51 different serotypes. HAdV viral infection is frequent, and before the age of 3 approximately 80% of all humans have been infected. HAdV infections cause approximately 5% of all colds and diarrhoeas in children. Viruses from subgroups A-C are causing most of these infections. Numerous diseases can be associated with HAdV including upper respiratory tract infections, acute conjunctivitis, cystitis and gastroenteritis. HAdV infections in immunocompetent people usually are harmless. However, HAdV has been discovered to play a role in the mortality of immunosuppressed patients, especially bone-marrow and stem-cell-transplant (SCT) patients appear to be at high risk for infection (7, 9, 11, 13). HAdV infections occur in 5 to 20% of stem cell transplantation patients and are associated with fatal disseminated infections in up to 60% of the cases (8, 12). HAdV serotypes 1, 2, 5, 11, 34 and 35 are frequently discovered in immunosuppressed patients and the disease often starts with an infection of the adenoids or the urogenital tract. It is not fully understood how the immune system responds to HAdV infections, but T-cell responses appear to be important for recovery from infection (8, 12, 23). There are no approved pharmacological treatments for immunosuppressed patients infected with HAdV-2, but the antiviral drugs Cidofovir and Ribavirin are effective in some SCT patients (1, 8, 10, 23).

The standard method for diagnosing HAdV infections has been viral isolation, but this method may require up to 3 weeks before a diagnosis can be established. In recent years PCR, which allows early detection of HAdV infection, has become the method of choice. By using a technique called real-time PCR it is possible to quantitatively detect all known serotypes of HAdV with high specificity and sensitivity, leading to a better and earlier diagnosis of HAdV infection (3, 8).

Conventional and real-time PCR

PCR (Polymerase Chain Reaction) is a technique by which any piece of DNA can be amplified quickly. It is a method for copying DNA, similar to the DNA replication that occurs in the cell prior to division (18). Each cycle of the PCR consists of three different steps: denaturation, annealing and extension. A pre-denaturation step can be included before denaturation. In each cycle, after the 60°C-step, the emission is recorded (Fig. 1).

Pre-denaturation (only the first cycle): Denaturation of the DNA to be amplified into single strands. Heating at 90-95°C about 5 minutes denatures the double-stranded DNA (the whole genome) making it single stranded.

Denaturation: Denaturation 90-95°C of the product from the amplification.

Annealing: The temperature is lowered to ~50-70°C and the primers can anneal to the denatured DNA. The primers serve as starting points for synthesising new DNA strands complementary to the target.

Extension: DNA-synthesis is carried out in 70-75°C. The Taq-polymerase, which is a heat stable DNA polymerase, extends the primers by adding nucleotides in the 5´ to 3´ direction, making a double-stranded copy of the target DNA (amplicon) (14).
The three above-mentioned steps comprise a cycle that can be repeated many times. The DNA-molecules are doubled with each cycle and the end-result is millions of copies of the DNA target region (amplicon).

**Conventional PCR:** In conventional PCR, post-PCR analyses must be performed for either size or sequence determination of the amplicon PCR-product. Hence, by conventional PCR you can only analyze the PCR-product at the endpoint. Gel electrophoresis is frequently used to measure the size of the amplicon. The mass and shape of the DNA products determine how far they migrate through the gel. However, this method cannot distinguish between molecules with about the same mobility (5). Hybridisation assays, where the DNA products are attached to the wells of a microwell plate, denatured and hybridized to specific probes, is an example of characterisation of the product by its sequence. Unfortunately, these multi-step procedures are time-consuming and there is always a risk of contamination.

**Real-time PCR:** Real-time PCR is based on the same reaction as conventional PCR, but the PCR-product is measured during each cycle and not only at the endpoint (Fig. 2). The amount of amplified product is measured through the detection and quantification of a fluorescent dye. The dye intercalates into double-stranded DNA (dsDNA) and does not bind to single-stranded DNA. When the dye is unbound in the solution it exhibits little fluorescence but when it binds to dsDNA it emits a strong fluorescence signal. The emitted light from this dye increases in intensity in direct proportion to the amount of amplified PCR product.
Fig 2. Amplification plot, HAdV-2, showing terms commonly used in real-time PCR.

During the initial cycles, there is no increase in the fluorescent signal, representing the baseline of the amplification plot. A fixed fluorescent threshold can be set above the baseline. The threshold, calculated by the computer, is based on the temporary background signals that occur before the amplification begins. The calculated threshold makes every signal that crosses the threshold significant. The variety of the baseline for each experiment makes each threshold unique and because of that a standard is always included making it possible to compare the different experiments. Ct (the threshold cycle) is the amplification cycle where the amount of amplicon rises above the threshold. With a high concentration of substrate DNA the threshold is reached after fewer cycles (lower Ct values) than with a low concentration of substrate. (5, 16) (Fig. 2).

A standard curve can be created by plotting the log of the initial copy number of substrate DNA molecules for a set of standards with known concentrations versus their Ct. By measuring Ct of an unknown sample and using the known standards in the standard curve the amount of DNA in the unknown sample can be quantified (16) (Fig. 3). The standard curve is created by plotting the log of the known target copy numbers of the different standards (1-10⁵) versus Ct. 100% amplification efficiency is the rate of amplification that leads to a theoretical slope of -3.32 in each cycle. This represents a duplication of the PCR product with each cycle. The acceptance criterion for the amplification efficiency, calculated by the program should be 90-110% (-3.1 ≥ slope ≥ -3.6) to be used as a standard curve. Curves from a serial dilution that differ with factor 10 should reach the threshold cycle (Ct) in intervals of 3.33 cycles for 100% efficiency which is optimal for a standard curve. The amplification efficiency (Eₐ) is calculated by the following equation Eₐ = 10⁻¹/slope -1.

Contamination is avoided and no post-PCR handling is needed in real-time PCR since the assays are performed in closed tubes, without any purification- or separation steps.
Nonspecific real-time PCR detection: There are different ways dyes can signal the amount of amplified DNA. YOPRO is a nonspecific dye that is added as a reagent in the PCR-mix. It exhibits little fluorescence when unbound in solution but emits a strong fluorescent signal when binding to double-stranded DNA (dsDNA). Fluorescent measurements monitoring the increasing amount of amplified DNA are performed at the end of the elongation step of each PCR cycle. Unfortunately, due to its non-specific properties, YOPRO binds to all dsDNA, including non-specific products and primer dimers. Hence, post-PCR assays may be required to provide a qualitative assessment of the final PCR product. Melting curves are useful for differentiating primer dimers from specific PCR-products. (Primer dimers usually melt at lower temperatures than specific PCR products.) Another useful method to identify primer-dimers, is agarose gel electrophoresis, which enables to correlation of product length to melting peaks.

The advantages of these non-specific detection dyes are that they are relatively cheap, as there is no probe-associated cost, and they can be used with any pair of primers for any target. This makes them very useful for optimizing primers in PCR reactions (2, 5).

Specific real-time PCR detection: For template-specific analysis the design and synthesis of a more custom-made fluorescent probe is required. There are several available specific chemistries (i.e. TaqMan, Scorpions, Molecular Beacons, Hybridisation probes) that all follow the same basic principle; a fluorescent signal is generated only when an amplicon-specific probe hybridizes to its complementary target. Non-specific amplification, due to mispairing or primer-dimers, does not generate a signal (2).

How do scorpions work?

Scorpions are sequence-specific probes that bind only to the target template, and they can therefore provide accurate and reliable quantification of the amount of target DNA (Fig 4). Scorpions combine both probe and primer in one molecule (unimolecular rearrangement), in a hairpin-loop configuration. Complementary sequences at both ends of the probe bind to each other forming the so called stem, which gives the probe its configuration. The molecule contains a 5' end reporter dye (fluorophore) directly linked to a quencher dye. A blocker, which prevents Taq DNA polymerase from extending the PCR-primer, is connected to the quencher dye. (18) When the scorpion is closed, i.e. in the absence of target, the quencher is located close to the fluorophore absorbing the emitted light (Fig 4.1). During annealing, i.e. in the presence of the
target, the hairpin loop unfolds and the probe binds to the newly synthesized target sequence that is complementary to the probe (Fig 4.2). The fluorophore and the quencher are now spatially separated which leads to an increase in the fluorescence emission. A significant increase in the fluorescent signal is detected by the real-time PCR instrument and it is directly proportional to the amount of target DNA (2, 5, 17).

Design of Primers and Scorpions

Two oligonucleotide primers (amplimers), specific for sequences flanking the target sequence, are designed. It is important to limit the possibilities for the primers to bind to any other regions in the DNA than those selected, i.e. the primers need to be target specific. There are a number of parameters to think about when designing these primers.

An optimal primer is approximately 18-25 nucleotides long with an even distribution of all four nucleotides to get an optimal melting temperature (Tm). The Tm is the temperature at which half of the primers are free and half are bound to their target sequences. Tm for the primers should be approximately 5°C above the annealing temperature. The GC content should be between 40 and 60 %, since the GC bonds melt at higher temperature than AT bonds. The Tms of the two primers in a primer-pair should not differ by more than 5°C (5, 23).

To test the efficiency of the designed primer pair, amplification using a nonspecific detection dye has to be performed.

The ideal amplicon length of the scorpion is approximately 100-200 base-pairs. A length of approximately 15-22 bases is ideal for the probe. This is to ensure fast and efficient binding of the scorpion to its target. The probe target should be as close as possible (2-50 bp) to either of the primers. A probe stem with 5-6 bases, containing mostly Cs and Gs, is most efficient for the scorpion and can be added as a separate stem to the probe or be induced in the probe. The 5’stem should not begin with a G since this may quench the fluorophore. The difference in the free energy, ΔG, between the closed and the opened scorpion should be more than 4 kcal/mol to ensure that the opened scorpion is preferred during annealing (60°C) (3, 17).
There are different computer programs that are helpful tools for primer design, e.g. primer3 (15) which is a free program from Massachusetts Institute of Technology (MA, USA), and Visual OMP version, 4.0.7.0. (DNA Software, US).

The aim of the project

Sangtec Molecular Diagnostics (SMD) is interested in commercialising a standardised real-time PCR kit for detection of HAdV. SMD develop and commercialize CE (Communauté Européenne)-labelled diagnostic tests in segments where there is a close relationship between treatment and diagnosis of disease, for instance diagnosing infections in immunosuppressed patients.

The aim of this study was to develop a prototype for a diagnostic assay for determination of HAdV, including the most frequent serotypes HAdV-1, 2, 5, 11, 34 and 35, in immunosuppressed patients. If the infection can be detected early, even prior to clinical symptoms, early initiation of antiviral drugs will hopefully reduce the mortality caused by this common virus in immunosuppressed patients.
RESULTS

Primer design

From the literature I found that the hexon gene in human adenovirus (HAdV) is highly conserved and thus has only minor sequence variations among the serotypes. It was therefore a suitable target gene for primer design. A sequence alignment of the hexon gene of HAdV-1, 2, 5, 11, 34 and 35, the most frequently discovered serotypes of HAdV in immunosuppressed patients was performed. The alignment showed that serotypes 1, 2 and 5 are identical in the selected target region. The partial sequences of serotypes 11, 34 and 35 are also identical, but differ from the first group (1, 2 and 5) in a few positions (Fig. 5). Note: Due to reasons of confidentiality the primers and probe sequences shown are not the selected sequences.

It is possible to design primers that work for all these serotypes, using degenerate primers with all nucleotide variants in the mismatching positions. Since HAdV-2 was the only variant from which DNA was relatively easy to acquire, this evaluation has focused on HAdV-1, 2 and 5 by using HAdV-2 DNA.

Fig 5. Alignment of the hexon gene of HAdV-1, 2, 5, 11, 34 and 35. Red bold letters show forward primer, blue underlined letters show reverse primer and green italic letters show probe target, here marked on HAdV-2.
* = Identical nucleotide in all serotypes.
Primers matching serotypes 1, 2 and 5 were designed by using Visual OMP software (version 4.0.7.0). One forward and one reverse primer were designed as a pair (for sequences see Fig. 5 and table 1). The melting temperature (Tm), was calculated by the program. The primers should have a Tm about 5°C above annealing temperature. Tm for the designed HAdV-2 forward- and reverse primer was 65°C and 64°C, respectively. This primer pair should work at a 60°C annealing temperature, which is a requirement for the diagnostic assay according to guidelines for Sangtec Molecular Diagnostics. For optimal amplification the PCR-product should be approximately 100-200 base pairs (bp) to ensure fast and efficient binding of the probe. The PCR product of these primers is 128 bp (Fig. 5). In Visual OMP I verified that the primers were unlikely to form primer dimers and that there were no secondary structures that might reduce the amplification efficiency.

**YOPRO evaluation of designed primers**

The efficiency of the designed primers was then evaluated with YOPRO using a serial dilution of HAdV-2 DNA ranging from 1-10⁵ copies per reaction (c/rx). The threshold was adjusted to 1000 dR (fluorescence units) according to design guidelines for the diagnostic kits.

![Amplification plot of the designed primer-pair from fig. 5. Serial dilution of HAdV-2 DNA ranging from 1-10⁵ copies/reaction were amplified. NTC is the Negative Control.](image)

The result from the YOPRO evaluation (Fig. 6) showed that the sensitivity of the primer-pair was 10 (c/rx) and the detection range was 10-10⁵ c/rx. To be able to detect even 1 c/rx, the Ct value of the 10 c/rx should be 37 cycles or less in each run. When using scorpion primers, the Ct values are usually reached a few cycles later as when YOPRO is used. Therefore, Ct values obtained with YOPRO should be as low as possible. If the Ct values are high the low sample concentrations could not be detected.

The negative control (NTC) included in the analysis was negative, showing that there were no problems with contamination or primer dimer formations. The Ct values were higher.
than what is desirable, and 1 c/rx was not detected. The amplification curves had the characteristic s-shaped form, showing that each PCR amplification was exponential (Fig. 6).

Fig. 7. Standard curve showing Ct versus log copies / reaction of the designed primer pair.
Serial dilution of HAdV-2 DNA ranging from 1-10^5 copies/reaction were amplified.
The amplification efficiency calculated by the program was 126.7%.

Looking at the standard curve of the dilutions 1-10^5 c/rx, the amplification had an efficiency of 126.7% (Fig. 7).
The YOPRO results were confirmed by gel analysis where a clear band was visible at the correct size of 128 bp. The analysis also confirmed that no primer dimers or other unspecific products were involved in the result (no data presented).

Improvements of amplification efficiency and sensitivity of the PCR

To improve amplification efficiency and sensitivity of the PCR, a number of variables can be changed. I tested different length of the amplicon, different primer Tm and also different primer sequences. Several dilution series of standards, ranging from 10-10^5 c/rx were prepared to ensure that the high Ct values were not due to a dilution mistake. Since the resulting amplification plots all showed the same results as in Fig. 6 and 7, the high Ct values were not caused by dilution problems with the HAdV-2 DNA. The PCR profile in this study could not be changed, since all the diagnostic tests must have the same PCR profile. DMSO and glycerol are adjuvants that denature the target DNA more completely and decrease primer and/or template secondary structure. They were used to improve amplification efficiency and get higher specificity (no unspecific products). The PCR conditions were also changed with different concentrations of magnesium chloride, primers, enzymes and nucleotides. Unfortunately, this extensive optimizing work did not result in any clear improvements.
Scorpion design

Even though the Ct values were high in the YOPRO evaluation I decided to continue with a scorpion (probe) design to see how much the high Ct values of the primers affected the efficiency of the scorpion. It was necessary that the probe was located near one of the primers because a short distance between primer and probe gives an efficient scorpion. The closest primer would then be included in the scorpion. As shown in the HAdV-2 sequence (Fig. 5), the chosen probe sequence was close to the reverse primer. Therefore the reverse primer was included in the scorpion. I used the Visual OMP software for the scorpion design (for sequence see table 1).

![Scorpion Design Diagram]

\[ \Delta G \text{ of stem (60°C)} = -2.20 \text{ kcal/mole} \]

Fig. 8. Probe in absence of target.
An example of a closed scorpion unbound to its target from the computer program Visual OMP.
Blue spots in the stem indicate AT-bonds and red spots indicate GC-bonds.
The probe has bound to its target during the annealing step. Blue spots in the stem indicate AT-bonds and red spots indicate GC-bonds between probe and target. The stem, primer and probe form a hairpin arrangement, which is typical for a scorpion primer (Fig. 8). The free energies of formation of the stem, ΔG, is shown in the figure.

The scorpion I used for HAdV-2 was labelled with FAM with an emission wavelength of 518 nm. The scorpion was closed in absence of the target so that the quencher DABCYL absorbed the emitted light of the fluorophore (Fig 8). During the extension step the scorpion became a part of the PCR amplicon. At the next cycle, in the denaturing step, the scorpion opened up and in the subsequent annealing step the probe hybridized to the target region. Figure 9 shows only 150 bp of the much longer amplicon. When the scorpion has opened up, the quencher and the fluorophore became separated, and the emitted fluorescence could be detected (Fig 9).

During the design, I took into account that the closed scorpion formation should not be so weak that it might open up in the absence of target, resulting in background emission. The free energy value of the closed scorpion, ΔG, should be > -1.5 kcal/mole to avoid background emission. Fig. 8 shows that the closed scorpion had a ΔG value of -2.20 kcal/mole. To favour the opened scorpion in the presence of target, the ΔG value should be at least 4 times lower than the ΔG value of the closed scorpion. I designed the opened scorpion to have a ΔG value of -7.78 kcal/mole (Fig. 9).
Scorpion evaluation

The designed scorpion was evaluated by amplification with the same HAdV-2 dilutions that were used in the YOPRO evaluation.

Fig. 10 Amplification plots of HAdV-2 with FAM labelled scorpion primer. Serial dilution of HAdV-2 DNA were used ranging from $10^1$ HAdV-2 genome copies / reaction.
Fig. 11. Amplification plots of Internal control (IC) with ROX labelled scorpion primer.
Serial dilution of HAdV-2 DNA ranging from $1-10^5$ HAdV-2 genome copies / reaction containing 1000 c/rx of IC each.

The sensitivity of the assay remained at $10-10^5$ c/rx. A comparison of the Ct values between Fig. 6 and Fig. 10 showed that the Ct-values obtained with the scorpion were a few cycles higher as compared to those with YOPRO, i.e. as expected. The amplification efficiency was still not acceptable (129.3%, no figure presented). Amplification plots using the scorpion primer did not have the s-shaped form characteristic for exponential amplification. This was most likely due to a weak probe binding of the scorpion, which was the only difference between the YOPRO and the scorpion evaluation.

The internal control, IC, was a 500 bp PCR fragment from *Drosophila* that was amplified in parallel with HAdV-2 in the same tube, but using a different scorpion primer (Fig. 11). It was used to test for false negative HAdV results and was always included in the diagnostic kits. The dye used in this study for the IC scorpion was 5-ROX (5-carboxy-X-rhodamine) which has an emission-wavelength of 602 nm. Since the dyes, ROX and FAM emitted light of different wavelengths, HAdV-2 and the IC can be measured simultaneously, in the same tube. In this reaction 1000 c/rx of IC was used for each dilution step of HAdV-2 and ROX should thus yield approximately the same Ct values for all HAdV-2 concentrations (Fig. 11). When IC is included in the reaction, it is possible that a competitive effect between IC and HAdV may cause a few cycles delay of the Ct values. However, amplification of HAdV-2 with or without IC showed approximately the same Ct values, suggesting that the IC had no competitive effect on HAdV-2 in this assay.

The scorpion result was confirmed by gel analysis in which clear bands were seen at the position estimated for fragments of 128 bp for the four highest concentrations ($10^{2-10^5}$ c/rx). No dimer bands or other products were visible (Fig. 12).
Evaluation with positive control

To evaluate if the low amplification efficiency and sensitivity were related to the use of HAdV genome DNA, a positive control consisting of a 128 bp optimal sequence for the primers was created. Compared to evaluations, in which the entire HAdV-2 genome was used, this short and optimal DNA sequence should have a sensitivity of 1 c/rx and amplification efficiency close to 100%. If the amplification efficiency was close to 100 % for 10-10⁵ c/rx the positive control could be used as a standard curve for quantification.
Fig 13. Amplification plot of positive control-scorpion primer. Serial dilution of DNA, 128 bp, from HAdV-2 ranging from 1-10^5 copies per reaction.

The result from the PCR demonstrated lower Ct-values compared to HAdV-2 genome and a sensitivity of 1 c/rx. However, 1 and 10 c/rx yielded the same Ct value and the amplification efficiency was 123%, which is far from 100% (Fig. 13).

**DNA sequencing**

To verify that the HAdV-2 viral DNA from Advanced Biotechnology (Columbia, US) had exactly the same sequence in the hexon gene region as the HAdV-2 sequence found in NCBI database that was used for the primer design (accession number AC_000007) DNA-sequencing was performed. A mismatch in the sequence can reduce the efficiency of the primers.

For the DNA-sequencing, a new primer-pair was designed outside of the first two primers. The new amplicon sequence was 358 bp and included the shorter 128 bp target amplicon. The result from the DNA-sequencing showed that the HAdV-2 DNA genome sequence was identical to the sequence from the NCBI database (no data presented).
DISCUSSION

Several studies have observed the serious problem connected with Human Adenovirus (HAdV) infections in immunosuppressed individuals, especially in stem cell transplant patients. This study is a first step on the way to commercialize a molecular diagnostic test for detection of HAdV. Since there are large diversities among the different HAdV-serotypes it would be very complicated and time-consuming to design primers and probes that would match all 51 serotypes. One of the most common serotypes observed in immunosuppressed patients is HAdV-2. Since HAdV-2 DNA was relatively easy to obtain it was chosen as the consensus sequence and primers that matched this serotype were designed. HAdV-1, 2 and 5 are identical in the selected target gene and therefore all these serotypes should be detected with the designed primers for HAdV-2. As the results show, one of the primer-pairs was optimized to a sensitivity of 10 copies per reaction (c/rx), which represents a good sensitivity. However, the amplification efficiency was not 90-110% with YOPRO which suggests that these primers could not be used for a quantitative test. The requirements for a molecular diagnostic test to be CE-labelled are high. A CE-labelled test has a very high quality and has gone through a lot of tests to ensure its sensitivity and reproducability.

Already at the beginning of the study, I encountered the problems with the efficiency of the primers. A sensitivity above $10^3$ c/rx often occurred, and many analyses contained primer dimers. Several different primer pairs were designed and evaluated with YOPRO in order to obtain a high sensitivity. In the study by Heim et al. (9) many serotypes were quantitatively detected by one primer-pair. I decided to test the same primers with YOPRO, but that resulted in a lot of primer dimers. The discrepancy might be due to chemistry differences (they used TaqMan probes). Therefore, I chose not to design a scorpion based on the Heim primers. Nevertheless, such a scorpion might have given good results since it appears to be YOPRO that in this study causes problems with unspecific binding and false positive results in this study (no data presented). I decided to design a scorpion from the most efficient primer-pair and the result from the evaluation demonstrated high sensitivity of 1 or 10 c/rx. However, the DNA was not exponentially amplified and the efficiency was far from 100%.

DNA sequencing

To evaluate if the reduced amplification efficiency was caused by a sequence problem, the amplicon was sent for sequencing. A mismatch in the sequence can reduce the efficiency of the primers. However, the sequencing result confirmed that the sequence from the amplicon used in the analyses was identical of that of the hexon gene region of HAdV-2 that was found in the NCBI database (accession number AC_000007) and used for primer design. This demonstrates that the primer problems are not due to mismatches in the sequence.

The amplification plots suggest possible reasons for the problems with the primers. A high Ct value often indicates ineffective primers and flat (not s-formed) curves can indicate inefficient scorpions. In this study there seemed to be problems with both.

Hypotheses for the low efficiency

The positive control fragment yielded somewhat lower Ct values. However, PCR using a DNA fragment as template should result in much better amplification efficiency. This showed that even under optimal conditions the primers did not perform well. There can be several explanations for this low efficiency. One possibility could be secondary structures of the HAdV-2 genome which would prevent primers from binding to the target. This could not be the only reason, however, since the efficiency was still low when using the control fragment as template.
A second possibility is that scorpion primers are difficult to design because the probe must be close to one of the primers and therefore the sequence of choice might not be the most optimal. It is also difficult to get the opened scorpion strong enough compared to the closed one and obtain a large energy difference between open and closed scorpion. Sequences in the loop are sometimes complementary to each other, which result in secondary structures lowering the efficiency of the scorpion. Due to this it is very hard to find a place in the genome that fulfils all this requirements.

A third possibility is that the primers and scorpions that I ordered from Advanced Biotechnology (Columbia, US) just have a purity of around 50%. When the primers have reached enough efficiency, i.e. have low Ct values and an amplification efficiency close to 100%, scorpions and primers that have a purity of at least 95% should be ordered from another company that have a purity of at least 95%.

**Conclusion**

Finally, my conclusion from all these experiments is that more work is needed on the primer design. New primers and scorpions have to be designed to get higher efficiency. However, a good start would be to first order the already designed primer with at least 95% purity. If this shows improved results a new scorpion can be designed.

When the scorpion for HAdV-2 yields good sensitivity with good amplification efficiency, most likely the serotypes that are identical in the target sequence should work with this scorpion too. The serotypes that have some mismatches in positions included in the target sequence can be degenerated in these positions. By doing this, a scorpion could be designed for all the serotypes in immunosuppressed patients.

Even though I did not have the time in this study to obtain efficient primers for all the serotypes I think that with more time a good primer-pair could be designed for all the serotypes that are common in immunosuppressed patients. This study is a first step of creating a commercial diagnostic test for HAdV that are essential to increase the survival rate for these patients.
### MATERIALS AND METHODS

#### Viral DNA material

Human Adenovirus serotype 2 (HAdV-2) viral DNA (50 ng/µl) was ordered from Advanced Biotechnology (Columbia, US). The DNA was tenfold serially diluted from 1 to $10^5$ copies/reaction (c/rx) in 10 mM Tris (HCl) pH 8.1, 0.1 mM EDTA, 0.09% NaN$_3$ and 20 mg/l Poly A. The conversion of ng to genome was based on the 35000 bp length of the Human Adenovirus genome. The dilutions were used as template in the PCR-reaction and were stored at -20°C until use.

#### Primer design

The genome sequence for the subtype HAdV -1,-2,-5,-11,-34 and -35 was found in the NCBI database subdivision “nucleotide”, FASTA format (17). The accession number for each serotype is AC_000017 (1), AC_000007 (2), AC_000008 (5), AJ272606.1 (11), AB052911.1 (34), AB052912.1 (35). Sequence alignment of the hexon gene region, 18000-20000 nt, was made by using the CLUSTALW software (18). HAdV-2 was used as a consensus sequence for the primer design using the program Visual OMP version, 4.0.7.0. (DNA Software, US). Table 1 shows primer pair sequences. The absence of homology of the primers with other relevant organisms was verified using BLAST (16). The primers were ordered from AtdBIO (Southampton, UK) at concentrations of 50 µM.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5’-ACAGTGCTGCACAGCCGTG-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-GTCCTAAGGATGCTGTTTTC-3’</td>
</tr>
<tr>
<td>Forward</td>
<td>5’-CAACGTGCCCTACCCACTAA-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-GGCAGCTGGTTGATGTTG-3’</td>
</tr>
<tr>
<td>Scorpion</td>
<td>5’- (1) CCGCGACGTTTAACGATTGACGCGG (2)(3)</td>
</tr>
<tr>
<td></td>
<td>ATTCCTCGGATGCTAGAAACAAGCAGCATCCTTAGGAC-3’</td>
</tr>
</tbody>
</table>

1Labels for Scorpion probes:
(1) = 6-FAM (6-carboxy-fluorescein), 5’-end reporter
(2) = DABCYL (4-((4-(dimethylamino)phenyl)azo)benzoic acid), Quencher
(3) = HEG (hexaethyleneglycol), Blocker
Evaluation of primer with YOPRO

PCR amplifications was carried out in 50 µl reaction volumes containing 25 µl of template DNA. PCR reaction mixes contained 1 µM YOPRO (Molecular probes, Eugene, US), 4 mM MgCl₂, 20 mM Tris (HCl) pH 8.1, 35 mM KCl, 15 mM (NH₄)₂SO₄, 0.2% Tween, 0.018% NaN₃, 2 mg/l Bovine Serum Albumin (BSA), 0.2 mM of each dNTP (G,A,C,U) 0.25 µM of each primer and 4U Fast Start Taq (Roche, Stockholm). For each analysis a sample with negative control, without DNA, containing 10 mM Tris (HCl) pH 8.0, 0.1 mM EDTA, 0.09% NaN₃, 20 mg/l Poly (A) was included. The real-time instrument used for the amplification was Mx3000P ™ (Stratagene, La Jolla, US). The amplification included one cycle of 37°C for 5 min and 95°C for 5 min, followed by 45 cycles of 95°C for 30 sec and 60°C for 1 min. In each cycle, after the 60°C-step, the emission was recorded.

10 µl of each PCR product was mixed with 2 µl 10 x loading dye (0.25% Orange G, 30% glycerol in water) and were loaded on a 2% agarose gel in 1 x TAE (40 mM Tris-HCl pH 7.7, 5 mM sodium acetate, 1 mM EDTA) containing 0.625 µg/µl ethidium bromide. The gel was run at 98 V in 1 x TAE buffer for 20 min and then examined and photographed under UV-light.

Scorpion design

A uniprobe scorpion primer was designed using the programe Visual OMP. For the sequence of the scorpion see table 1 (scorpion primer). The scorpion was ordered from AtdBIO at a concentration of 50 µM. PCR amplifications were carried out in 50 µl reaction volumes. PCR reaction mix contained 1 µM YOPRO, 4 mM MgCl₂, 20 mM Tris pH 8.1, 35 mM KCl, 15 mM (NH₄)₂SO₄, 0.2% Tween, 0.018% NaN₃, 2 mg/l BSA, 0.2 mM of each dNTP (G,A,C,U), 0.25 µM forward primer, 0.25 µM scorpion primer, 0.1 µM IC (Internal Control) primer, 0.1 µM IC-scorpion primer, 4U Fast Start Taq and 2U UDG (Uracil-DNA glycosylase). 25 µl of template DNA was used. A negative control containing 10 mM Tris (HCl) pH 8.0, 0.1 mM EDTA, 0.09% NaN₃, 20 mg Poly (A) was included in each analysis. Amplification consisted of one cycle of 37°C for 5 min and 95°C for 5 min, followed by 45 cycles of 95°C for 30 sec and 60°C for 1 min. In each cycle after the 60°C-step the emission was recorded and evaluated.

After amplification, 10 µl of each PCR-product, mixed with 2 µl 10 x loading dye, was loaded on a 2% 1 x TAE agarose gel as described earlier.

Generation of positive control

PCR amplification was carried out in 50 µl reaction volume containing 25 µl template DNA (4000 genome/µl HAdV-2). PCR reaction mix contained 4 mM MgCl₂, 20 mM Tris (HCl) pH 8.1, 35 mM KCl, 15 mM (NH₄)₂SO₄, 0.2% Tween, 0.018% NaN₃, 2 mg/l BSA, 0.2 mM of each dNTP (G,A,C,T) 0.25 µM of each primer (table 1) and 4U Fast Start Taq. The same instrument and amplification steps as before were used. The PCR product was purified using QIAquick PCR Purification Kit (QIAGEN). The eluted 128 bp PCR fragment was loaded on a 2% 1 x TAE agarose gel as described earlier. 10-fold serial dilutions from 1 to 10⁵ c/rx were prepared of the PCR fragment. The dilutions were used as template in the PCR reaction and were stored at -20°C.

Sequencing of viral DNA

For the primer design the freeware Primer 3 (15) was used. Primer sequences are shown in Table 1. The primers were ordered from AtdBIO at concentrations of 50 µM. PCR amplification was carried out in the same way as described in generation of positive control. The amplicons were
purified on 2% 1xTAE agarose gel using QIAquick PCR Purification Kit (QIAGEN) to eluate DNA from the gel.

The purified sample was subjected to a new gel electrophoresis (2% 1xTAE agarose). 10 µl (20 ng/µl) of the PCR product and 10 µl (50 µM) of each primer were sent to KIseq (Karolinska Institutet, Stockholm) for sequencing.
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THANKS!
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