

Genomic screening of high frequency antigens in blood donors

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Background

All red blood cells bear different blood group antigens on the cell surface which are carried on different glycoproteins and glycolipids. As blood becomes transfused to another person, the recipient's immune system can respond and in some individuals, antibodies are produced against the antigens of the donor's blood cells. Some blood group antigens occur as high frequency antigens, that is, they are found in ~99% of the population. Thus, transfusion of patients with antibodies to a high frequency antigen is dependent on one thing: finding sufficient compatible blood. When such a patient needs to be transfused, it is too late to start screening due to the unfavorable odds of finding the appropriate antigen-negative blood. Screening should be a preemptive measure in larger blood banks to avoid these situations.

Study design and methods

A multiplex PCR with sequence specific primers (SSPs) was designed and optimized to detect alleles encoding 6 different high frequency antigens. DNA was amplified directly from a whole blood lysate using the Extract-N-Amp™ Blood PCR kit. All negative samples were confirmed either serologically or by DNA sequence analysis.

Results

35 donors with various rare phenotypes were discovered in a screening process of 1799 donors from the southern part of Sweden.

Conclusion

The multiplex method required considerable optimization of both PCR product size and stability but in the end a robust method was developed that proved economically viable. Each donor test is very cost-effective since multiplexing permits 6 typings in one reaction at a cost well below that of conventional serologic tests. The method has potential also to be used in more automated analysis such as fragment analysis and direct sequencing.

All red blood cells have functional molecules on the cell surface. They act, for example, as ion channels, transporters, adhesion molecules or simple glycolipid structures. Many of these carry polymorphisms which are recognized as blood group antigens. When blood becomes transfused to another person, the recipient's immune system may react by producing antibodies against the antigens of the donor's blood cells. This process takes a couple of days to weeks. The immune response to blood varies between individuals, some receive 100 blood units without producing antibodies while others receive 1 unit and form antibodies.[1]

If the immune system has produced an antibody against a blood group antigen, it will react if this antigen is encountered again; potentially resulting in various degrees of immediate or delayed transfusion reactions, some being so severe that if left untreated could seriously harm or kill a patient.

The different antigens on a red blood cell are divided into one of 33 different blood group systems, of which ABO and Rh are the most well known. Some antigens in blood group systems are polymorphic, others are found either as high frequency antigens because they are found in ~99 % of the population, or as low frequency antigens (found in less than 1% of individuals). Generally high frequency antigens do not cause any harm since the recipient is most likely also positive for the antigen. But what if a patient lacks one or

more of these high frequency antigens and requires blood?

The possibility to transfuse patients with antibodies to high frequency antigens is dependent on finding compatible antigen-negative blood. When the need arises for such a patient to be transfused, it is too late to start screening donors because the likelihood of finding compatible blood is less than 1 in 100 typed depending on the antigen. [2] Consequently, this scenario usually has two outcomes unless antigen-negative donors are known: to cancel the pending operation or to delay the transfusion in order to obtain appropriate compatible blood from other blood banks or the WHO Rare Donor Registry, which may take several days. As the blood unit is rare, it is most commonly stored frozen, mixed with glycerol, in liquid nitrogen (-195°C) or in a -80°C freezer. When blood arrives, it needs to be washed and resuspended, which in the end leads to a blood unit with less effect compared to a fresh blood unit. Seltsam *et al.* reviewed current practice in Germany, Austria and Switzerland and concluded that about one third of patients with high frequency antibodies received unsatisfactory transfusion therapy. [3]

Similar to hemolytic disease of the newborn (HDN), antibodies against platelet antigens is the most common cause of fetal and neonatal alloimmune thrombocytopenia (FNAIT). Platelets are usually much harder to work with since they have a short lifespan, phenotyping is usually employed by flow cytometry or monoclonal antibody immobilization of platelet antigens (MAIPA), which is very time-consuming. Blood centers need to have an inventory of human-platelet-antigen (HPA)-negative platelet concentrates in case of a FNAIT.[4]

Most blood banks do not have a typing program to identify donors that lack high frequency antigens and other rare antigens. There are few commercially available antisera thus forcing blood banks to use sera from immunized patients, which is unstandardised and of limited supply. Patients' sera also tend to deteriorate upon storage and large volumes are needed for mass typing. As the sera deteriorate, there is a risk of mistyping donor RBCs, denoting them antigen-negative when they really are weakly antigen-positive thus increasing the risk of delayed hemolytic transfusion reactions. [5]

The aim of this project was to develop an effective and low-cost method to identify blood donors with rare blood groups. There are several high frequency antigens that are clinically relevant and for which, screening for the antigen-negative phenotypes would be an advantage in a large Blood Centre. However traditional serological methods are restricted as described above. Since the molecular basis of most blood group antigens is known, it is possible to decide the phenotypic expression of patients/donors blood group by simple PCR methods. However, current methods of DNA preparation demand much "hands-on" time and several steps; such as lysing cells, removing unwanted byproducts and precipitating DNA in alcohol. Thus, we set out to develop a multiplex-PCR that takes as few steps as possible yet giving a cost effective and safe result, starting from simple whole blood and move directly to PCR amplification and gel separation.

The antigens investigated in this project are Co^a (1:500), Kp^b (1:1500), Sc1 (1:269000), Yt^a (1:1000), HPA-1a (1:100) and Vel (1:1250). Numbers in parentheses indicate the prevalence of the antigen-negative

phenotype. These antigens were picked based on demand in our institution and are connected to the northern European population but this method can be used for others as the molecular basis of most blood group antigens are known. [2, 6, 7]

Blood group genotyping is already a common practice in many blood centers and larger Transfusion Services, but only used in cases where serological typing is impossible due to multiple transfusions or problematic serology as a low-throughput method. [8-10]

As stated by Avent *et al.*, genotyping is already competing with serological methods and is becoming routinely used. Though the more common serological typing of ABO and Rh is favored due to its fast pace, the typing of high frequency antigens with SSPs in a multiplex PCR can produce large amounts of data with a minimal input of labor. For instance, manually typing six high frequency antigens per donor and 110 donors a day nets a result 660 typings done, a feat which can be improved even more if the process is automated.[11]

The southern blood banks of Sweden normally receive about 200 to 250 donations each day where 1 rare blood unit could be procured every day without knowing it. Designing a mid-throughput method of genotyping these high frequency antigens could potentially solve smaller blood banks issues in identifying rare antigen-negative blood.

Materials and methods

Samples

Blood samples (O RhD +/-) in anticoagulant citrate dextrose (ACD) solution from donors

in Skåne were collected within two weeks of donation. Each sample was controlled so that no previous screening was performed. 10 µl whole blood was transferred to an eppendorf tube and frozen for at least 1 day. DNA preparation was performed with Extract-N-Amp™ PCR kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. The whole sample was mixed in with 20 µl lysis buffer. After five minutes incubation, 180 µl neutralization buffer was added and the samples were used the same day. Samples could be kept in +4 C for at least a week but it was seldom necessary.

DNA preparation using a high-salt method

DNA from samples needing sequencing as confirmation was prepared with a high-salt method: 400 µl of whole blood was frozen overnight and then washed with a lysis buffer until no hemolysis could be seen.

The leukocyte pellet was digested with 1 mg of Proteinase K in 0.5% SDS/2 mM Na₂EDTA for 1 hour at 55 °C. 200 µl 6 M NaCl was added and mixed vigorously to extract the proteins in the samples, then centrifuged for 2 minutes at 14680 rpm. The eluate containing DNA was precipitated with 1 ml 95% ethanol, washed with 1 ml 70% ethanol and dried for 1 hour at 55 °C. The final pellet was dissolved in sterile water, the concentration determined using a Nanodrop spectrophotometer and then diluted to a concentration of 100 ng/µl.

PCR

A PCR primer mixture was made, containing all necessary primers (See table 1) for *CO*A*, *KP*B*, *SC*01*, *YT*A*, *HPA*1a* and *VEL*, cresol red and 25 mM MgCl₂. Two

ul of the sample lysate were pipetted into 8 ul of the PCR primer mix and 10 ul Extract-N-Amp PCR Mix. This was amplified by the following program.

Initial denaturation at 94°C for 6 minutes; 10 two-step temperature cycles: 94°C for 10 seconds followed by 66°C for 45 seconds; 30 three-step cycles: 94 °C for 20 seconds, annealing at 66°C for 30 seconds and elongation at 72°C for 45 seconds. The thermal cycling program was completed with a final elongation at 72°C for 7 minutes.

Analysis

The PCR products were separated by electrophoresis on a 3% agarose gel containing Sybr® Safe (Life Technologies, USA, a less hazardous alternative to ethidium bromide), in 0,5% TBE buffer. After separation the gel was photographed under UV illumination.

Each batch of PCR master mix was tested with control DNA samples homozygous for each of the antithetical low-frequency alleles (see figure 1).

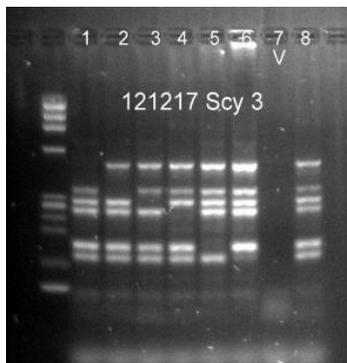


Figure 1 shows the PCR reaction controlled with a DNA sample homozygous for each of the antithetical low-frequency alleles (lanes 1-6), followed by a water control (lane 7) and a normal donor sample prepared with the Extract-N-Amp™ kit (lane 8). The DNA ladder is shown to the left of the marked lanes.

As the likelihood of finding a negative donor for all six antigens is less than 1 in a trillion ($1:2.52 \cdot 10^{19}$), no negative control is needed. A water control was included in each PCR. If a specific band was absent from a donor sample, complementary serological investigation or sequencing was done.

Sequence analysis

The only allele needing confirmation by sequencing was *HPA*1a* which was done with a general PCR amplification (see table 1 for sequencing primers) from salt prepped DNA and elution with Qiagen Gel Extraction Kit following the standard protocol. The product was amplified with BigDye® Sequencing Mix v3.1, then precipitated with 60 µl 95% ethanol and 5 µl 125 mM EDTA. The precipitate was washed with 250 µl 70% ethanol and dried in a DNA SpeedVac. Band sequencing was performed on an Applied Biosystems Genetic Analyzer 3500 DX according to the manufacturer's specifications.

Serological confirmation

The commercially available reagents, anti-Kp^b (BioRad, USA) and anti-Co^a (Immucor, USA) were used for serological confirmation. Typing for Co^a was done with IAT tube method, 1 drop antisera and 1 drop 3% blood suspension, incubation for 30 min at 37°C followed by 3 washes in PBS and addition of 1 drop antihuman globulin.

As no Kp(b-) samples were found, serological typing was never performed. The method of choice would be the standard IAT gel technique, using cards containing anti-IgG/-C3d in which 25 µl anti-Kp^b and 50 µl 0.8% blood suspension diluted in low ionic saline solution (LISS).

The card would then be incubated for 15 minutes at 37°C followed by 10 min centrifugation.

The other three antigens were confirmed with noncommercial anti-Vel, anti-Yt^a and anti-Sc1 from our collection of rare sera using the IAT gel technique as described above for anti-Kp^b.

Results

Of the 1799 donors screened, 35 were found to lack a high frequency antigen investigated in this project: two Vel-negative, two Co(a-) and 31 HPA-1a-negative.

Originally *LU*B* was included in the mix instead of *SC*1* but due to poorly reproducible PCR amplification, it was replaced with *SC*1*.

144 samples were screened with the first mix containing *LU*B* primers, 843 with a mix without *LU*B* and 812 with *SC*1* primers.

We felt that the presence of *LU*B* primers were inhibiting the other primer pairs and, by removing *LU*B*, the success rate for each amplification was increased from 86.8% (19 failed amplifications of 144 samples) to 96.8% (53 of 1655 samples).

Not every antigen followed the expected frequencies, no Yt(a-) and Kp(b-) samples were found in this project when at least 1 was expected (see table 2). The remaining antigens followed the expected frequency.

One sample lacked the CO*A band but gave varying results in the serological confirmation. The commercial reagent gave a weak positive reaction while two different patients' anti-Coa were non-reactive. Sequencing was performed and the sample had the signature 134C>T mutation that results in a Co(a-) phenotype. A control sample has been requested.[2]

Work time and costs

The average time for the work process is 3 hours and 15 minutes with a hands-on time of 1 hour 30 minutes. The actual work routine was: Thaw three batches (3 x 36) of previously frozen samples, gather 36 new samples, check the current phenotype, mark tubes and transfer 10 µl blood to an eppendorf tube. Freeze the new batch while preparing the thawed samples and start the thermal cycling process for one batch. During the cycling, a large agarose gel was cast and a new batch of donors were gathered, controlled and frozen. As the PCR ends and the samples are put through gel electrophoresis (see figure 2), the remaining two batches are amplified. Optimization showed that it was important to start the amplification promptly after the PCR reactions were prepared and that it could be improved with a multichannel pipette. Therefore PCR was performed in batches of 36 samples.

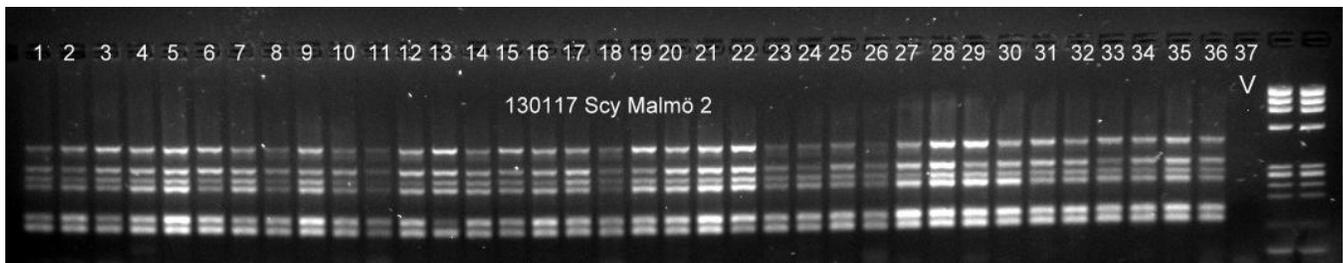


Figure 2 - A typical batch of screening donors with a water control in lane 37.

As the second and third batches amplify there is ample time to gather and finish the third batch. When the last two batches are amplified, they are separated by electrophoresis and the first completed batch's results are put into the database. A more detailed flowchart can be found in the supplemental info (see the flow chart in Figure 7).

The cost of typing donors just based on reagents is 35 Kr (3,99€) per sample. For more detailed cost analysis, see tables 3 and 4.

Optimization considerations

Although the process was time-consuming in the beginning to find the appropriate concentration of each primer, it was well worth the optimization. Most notable effects when using Extract-N-Amp™ is the high annealing temperature needed for amplification of the products. A difference of 1 degree Celsius was critical to the process.

Gels with different DNA markers were tested as well. The DNA dye Sybr® Safe was compared with GelRed™, but GelRed™ gave too great a signal even in at low dilution of either dye or amplicon (see figure 3).

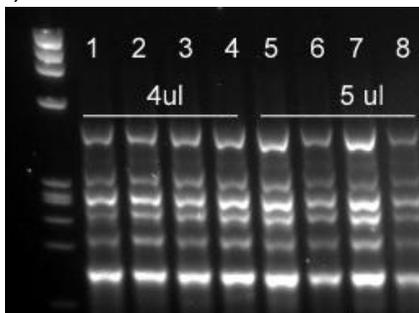


Figure 3 - GelRed™ gel with 4 and 5 µl DNA in each well. The appearance of "bird wings" is due to the rough DNA preparation.

Different agarose concentrations were examined as well, too low (2%) or too high (4%) agarose gel resulted in a separation that took too long or gave poor resolution (see figure 4 and 5).

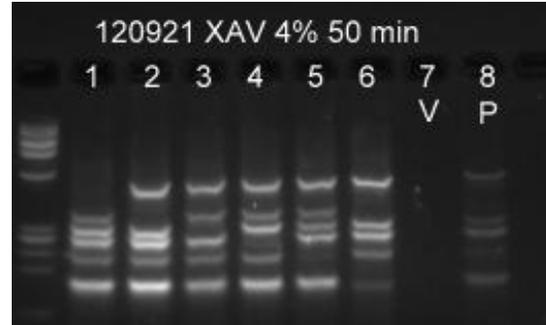


Figure 4 - DNA controls with the primer mix without LU*B and SC*1 on a 4% agarose gel dyed with Sybr® Safe after 50 minutes of electrophoresis at 160V.

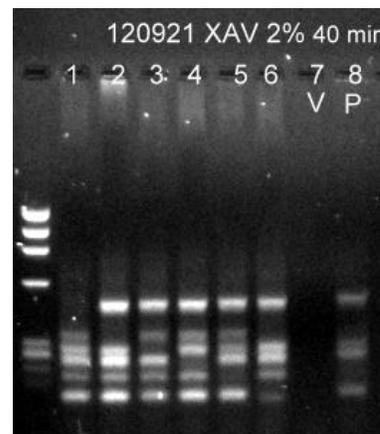


Figure 5 - Same content as seen in figure 4 except 2% agarose gel instead of 4% and 40 minutes of electrophoresis at 160V.

This process can be compared with Thermo Scientific's Phusion kit which is based on a similar idea. Phusions recommendation is to add 1 µl whole blood straight to the PCR tube and amplify with a denaturation temperature of 98°C for 1 second and a variation of annealing and extension temperatures for about 15 seconds/kb. While the amplification program is short there is a greater risk of nonspecific primer binding due to leftovers from blood.

The DNA “extraction” with Extract-N-Amp™ also leaves remnants although much more diluted. We also found that donors with lipemic plasma were not suitable for DNA extraction with this method, the lysing buffer seemed not to be able to lyse enough resulting in a brownish tinted sample instead of a more orange/red sample.

Discussion

The aim of this project was to design and optimize a multiplex screening protocol for high frequency antigens to suit the needs of our Blood Centre. Other blood typing methods that use microarrays, eg. Beadchip, have large multiplexing capabilities but have longer analysis time. Hashmi *et al.* [12, 13] used a semiautomatic protocol for DNA extraction of 96 donors which took 4 hours. By that time, at least 36 donors would be completed using the Extract-N-Amp™ method. While the Beadchip detects 24 different antigens, it has a price that most smaller blood centers cannot afford. Montpetit *et al.* [14] worked on a SNPstream® platform (Cologne Center for Genomics, Germany) with a similar approach to Hashmi *et al.* but their analysis took three days although the sample requirement for DNA preparation was only one drop of blood. Denomme and Van Oene [15] used the same approach as Montpetit *et al.* but focused on 12 common blood group SNPs.

Extract-N-Amp™ requires a Thermal Cycler and gel electrophoresis equipment, which are standard genotyping equipment. An array method can process several plates in parallel which makes it a suitable method in larger blood centers while Extract-N-Amp™ is suitable as a medium throughput method. [12-15]

One potential disadvantage of multiplexing is that when you are searching for a particular phenotype you waste reagents typing for the other blood groups. While this is no major concern in PCR genotyping due to the low cost of primers, a microarray, such as those described above, is specifically designed and minor modifications are not possible. A new microchip is then needed resulting in a more laborious work than just making a new mix with fewer primers. Karpasitou *et al.* [16] gave a price estimate of less than \$3 per SNP per sample, not including DNA preparation and machine costs. While the price of Extract-N-Amp™, using the method described here, is roughly \$5 per sample not including machine costs, it is possible to minimize costs even further to less than \$3 according to Wagner *et al.*[17]

Typing for blood group antigens using DNA methods also has the disadvantage of only being able to report the typical (expected) variant of the blood group. Rare or new nonsense mutations resulting in an antigen negative phenotype will be seen as antigen positive in DNA typing methods. [18] For instance, the Co(a-) phenotype could be a product of three different missense mutations, the most common being the 134C>T screened for in this project. The other two, 133G>A and 140A>G, will result in a Co(a+) phenotype in this method, and this is not even including the other 6 different silencing mutations.[2] While rare null alleles might be mistyped, patient safety is never compromised due to the serological confirmation.

Small trial runs using the Extract-N-Amp™ DNA in direct sequencing were also performed instead of using high salt-prepared DNA. While it was possible to

obtain decent data, the repeat rate was very high, 9 failed runs of 22 instead of the normal rate of 10 fails of 57 with salt prepared DNA. Lee *et al.* had more success by using AnyDirect and purifying with Qiagen Gel Extraction Kit and sequencing with BigDye® Terminator Cycle Sequencing Kit. This indicates that it is indeed possible to sequence individual samples relatively quickly.[19]

Fragmentation analysis was also tested on the Applied Biosystems Genetic Analyzer 3500 DX with FAM-labeled primers for *HPA*1a*, *YT*A*, *VEL* and *CO*A* and it gave a promising result (see figure 6 in supplemental info). Large peaks were seen at the different amplicon products but due to the rough DNA preparation there was much background noise. Diluting the samples prior PCR amplification could solve this problem.

Of the donors screened, there was no Kp(b-) (1:1500) Yt(a-) (1:1000) or Sc:-1 (1:269 000) donor found. While too few donors were screened for Sc1, statistically at least one Yt(a-) and one Kp(b-) donor should have been found in this study, however the numbers of donors screened is still relatively small. The controls for the mix were prepared using the high salt method resulting in a much cleaner DNA product but a Yt(a-) donor was tested using the Extract-N-Amp™ method as well. No discrepancies could be seen between the different DNA preparation methods.

While optimizing the method, it was hard to achieve a negative water control. Weak bands could be seen in one water sample while the next one was negative. As the PCR-ReadyMix is amplifies everything it can, sterile handling is an absolute must if negative water controls is to be attained.

The amount of clean DNA needed for amplification was as low as 15 ng.

Improvements can be made in this method; much of the time is spent on transferring material between tubes. In the absence of a fully automated robot, a simple multichannel pipette could dramatically decrease the time spent on sample and PCR preparations.

A similar approach to that of He *et al.* [20] would be interesting to test as well using the Extract-N-Amp™ method. This group pools DNA samples in search of low frequency antigens using a multiplex PCR. While the pooling might not be necessary due to the already favorable price, multiplex PCR scanning low frequency antigens with a positive control such as human growth hormone could benefit blood centers.

In conclusion, we have shown that it is possible to develop a multiplex PCR capable of phenotyping for six high frequency antigens for relatively low cost using standard PCR equipment. Based on the results of the project, a screening program has been implemented in the Blood Centre to continue to screen donors and to identify blood for immunized patients in need of these rare units.

Acknowledgements

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Supplemental info

Table 1 – Primer sequences, concentrations and products

Antigen	Forward Primer	Reverse Primer	Concentration per primer	Product (bp)
<i>KP*B</i>	CTCTTCCTTGTC AATCTCCATCACTTTAC	CGAACCTCTGCTTTAGCAGCATC	0,35 pmol/L	493
<i>HPA*1a</i>	ACTTACAGGCCCTGCCTCT	TTCTCTTGCCCACACCTCCCC	0,25 pmol/L	352
<i>YT*A</i>	GAGCGGTGCCTCCAATGGAC	CCACTAGTTACCTGCAGGTTGTG	0,25 pmol/L	303
<i>VEL</i>	CGGAGTCAGCCTAGGGGC	CTGGGCGCTCTGCTGGAG	0,2 pmol/L	266
<i>CO*A</i>	TGCCAGCATGGCCAGCGAG	ACATCTTCACGTTGTCCTGGAACG	0,3 pmol/L	164
<i>SC*01</i>	CTCCCAGTTGGCCTTGTCTC	TCACCTCCTTGGGTACCGTACC	0,35 pmol/L	138
<i>HPA*Seq</i>	GGCCTGCAGGAGGTAGAGAGTCGC	TTCTCTTGCCCACACCTCCCC	0,3 pmol/L	415
<i>CO*Seq</i>	GTGACAGCCGGGTTGAGGTG	TGCCAGCATGGCCAGCGAG	0,3 pmol/L	246

Table 2 – General information on the screened blood groups

Antigen	Gen	Mutation	Expected frequency	Observed frequency
Kp ^b	<i>KEL</i>	961C>T	1	0
HPA-1a	<i>GPIIIa</i>	186T>C	18	31
Yt ^a	<i>ACHE</i>	1057C>A	1	0
Vel	<i>SMIM1</i>	Not published	1	2
Co ^a	<i>AQP1</i>	134C>T	3	2
Sc1	<i>ERMAP</i>	169G>A	0	0

Table 3 – Overview of the work process and time spent

Worksteps	Time (Bold = Hands-on Time)
Gathering samples	6 min
Marking tubes and protocol, mixing samples	35 min
DNA Prepping	10 min
Pipetting in PCR tubes	20 min
Casting gel (for 120 samples)	8 min
Pipetting in gel	10 min
Photographing	1 min
Thermal Cycling	1 h 45 min
<i>Total time</i>	1:30/3:15 min

Table 4 – Analytical costs

Consumables	Price per analysis
DNA Stain (Sybr® Safe)	0.275 Kr (0.03€)
Tubes	1 Kr (0.11€)
Primers	0.7 Kr (0.08€)
Extract-N-Amp™ Blood PCR Kit	23 Kr (2.63€)
Filter tips	4 Kr (0.46€)
Agarose	3 Kr (0.34€)
TBE buffert	3 Kr (0.34€)
Total	34.975 Kr (3.99€)

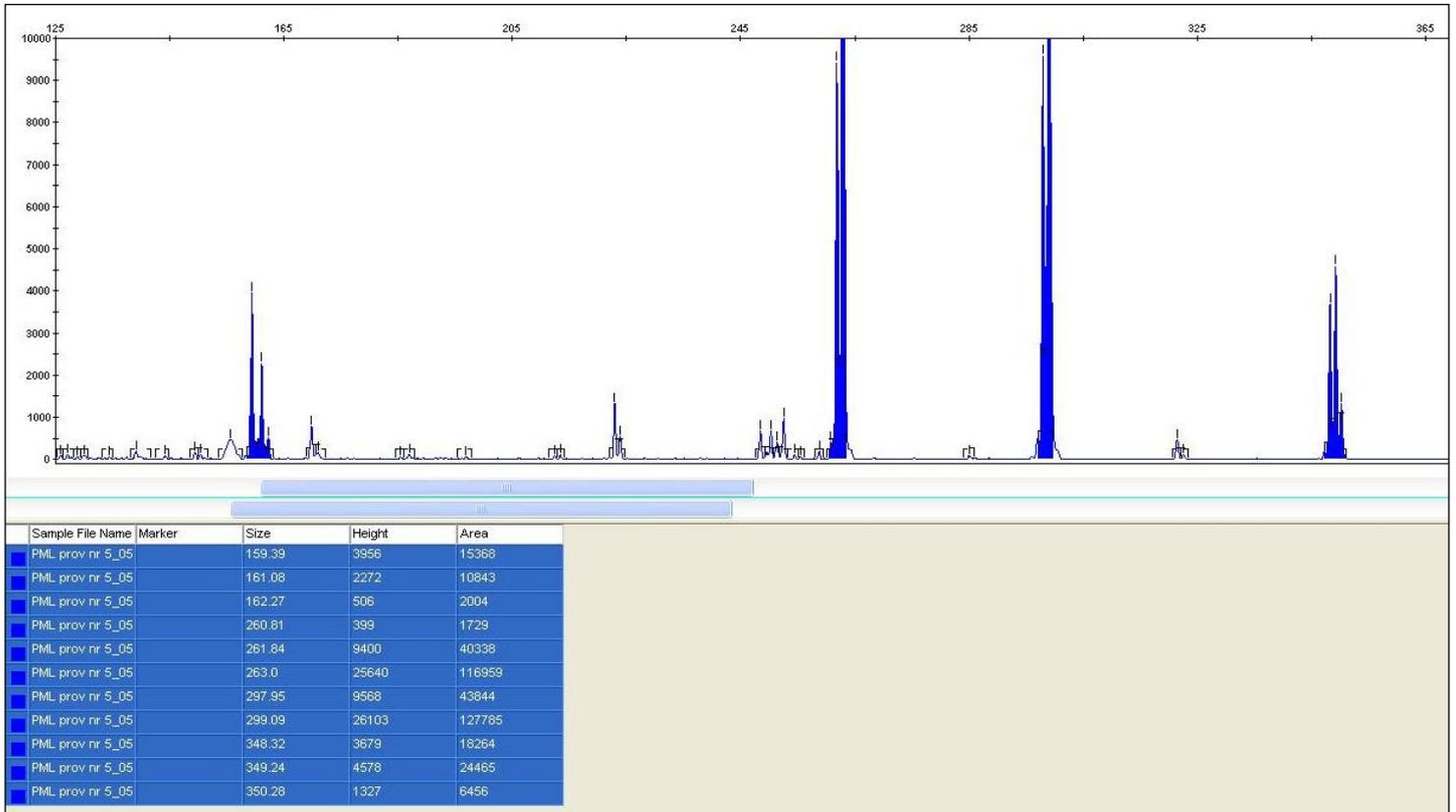


Figure 6 - The peak diagram from an Extract-N-Amp™ sample. The primers would give products of 164 bp (CO*A) 266 bp (VEL) 303 bp (YT*A) and 352 bp (HPA*1a). The slight miss in the resulting peaks could be due to “stutter” peaks, in which different amplicons of the expected product can differ by two or four base pairs.

Figure 7: Flow Chart showing the workflow of sample preparation, amplification and analysis

