Automated DNA extraction:
Integration of the chemagic Magnetic
Separation Module I with the Perkin Elmer
MultiProbe II Liquid Handler

– *Technology Assessment Report* –

December 2008

Revised April 2009

Contributors

Name	Laboratory	City	Country
Tom Janssens	Center for Human Genetics	Leuven	Belgium
Ivo Salden	Center for Human Genetics	Leuven	Belgium
Gert Matthijs	Center for Human Genetics	Leuven	Belgium

Contact information

For questions or an electronic copy of this report please contact the first author:

Tom Janssens
Laboratory for Molecular Diagnosis
Center for Human Genetics
University Hospital Leuven
Herestraat 49
B-3000 Leuven
Belgium

e-mail: tom.janssens@uzleuven.be
phone: +32 16 34 16 97
fax: +32 16 34 60 60

Technical Support

chemagen

website: www.chemagen.de
Or contact local chemagen representative.

Perkin Elmer

website: www.perkinelmer.com
Or contact local Perkin Elmer representative.

Conflict of Interest

The authors state that there has been no financial conflict of interest.

Abstract

DNA extraction is a key factor in molecular genetic testing. Automation of this process has some important benefits, including increased throughput, more consistent and reproducible processing and improved sample tracking. Manufacturers have produced a plethora of platforms, with different extraction chemistries. However, the extraction of large blood volumes (>1 ml) is still a challenge, for which only a few systems are available.

This EuroGentest project involves the assessment of the chemagic Magnetic Separation Module I (chemagic MSM I) extraction robot (chemagen), integrated with the Perkin Elmer Multiprobe II (PE MPII) liquid handling robot. The extraction technology is based on the use of paramagnetic beads with a DNA binding coating. Using magnetic rods, these beads can be transferred from one DNA washing buffer to another when applying an electro-magnetic field.

The aim of this validation was to describe in detail the physico-chemical qualities of the extracted DNA. In addition, we wanted to compile a generic Standard Operating Procedure (SOP) for the use of this integrated system.

Therefore a study was performed, including over 180 anonymized whole blood samples on EDTA. The DNA concentration was determined with a spectrophotometer and the yield was determined in relation to the white blood cell count. The 260/280nm ratio was calculated as a parameter for the quality of the DNA. In addition, all DNA samples were included in a multiplex drop-out PCR to check their initial performance. Furthermore, samples were directly loaded on agarose to detect possible early degradation of the DNA. Stability of the DNA was evaluated by repeating the multiplex PCR and the degradation test on randomly selected samples, stored at different conditions, during several months. Finally, the performance of the extracted DNA for some in-house downstream diagnostic tests was assessed.

Our study has shown that the integrated chemagen-Perkin Elmer DNA extraction platform is an easy-to-use, flexible system resulting in DNA of good quality, with a high yield and a satisfying performance for diagnostic purposes.

1. Introduction

The majority of molecular genetic tests start with the extraction of DNA, indicating the great importance of a suitable and reliable method for this purpose. Next to many in-house standard operating procedures (SOPs), a lot of commercial extraction kits have been developed. These methods are generally accepted and have proven their validity with good results.

However, it is obvious that the automation of the extraction procedure could have major benefits, i.e. a higher throughput, more reliable and reproducible processing of the samples, improved traceability and possible streamlining of the entire DNA extraction procedure. Ever since this became clear to the molecular genetics community, a plethora of platforms, using different extraction chemistries, were produced by different manufacturers. The most common chemistries for this purpose include salting-out extraction, solid phase extraction and extraction with paramagnetic beads.

For routine diagnostic use most DNA is extracted from white blood cells in whole blood samples. This can either be done for small (< 1.5 ml) or large (> 1.5 ml) volumes of blood. The latter is the most challenging with only a few automated systems available on the market. The chemagic MSM I extraction robot (Figure 1) from chemagen was selected as the first automated extraction platform to be validated within the context of the EuroGentest network. In order to streamline the entire process as much as possible, a Perkin Elmer Multiprobe II liquid handling robot was integrated with the chemagic Magnetic Separation Module I extraction robot. Both robots are considered as one integrated DNA extraction platform and thus included within the scope of this validation project.

The aim of this project was to describe in detail the instrument specifications and the physico-chemical quality of the extracted DNA. In addition, we wanted to assess the general performance of the resulting DNA in a variety of downstream molecular diagnostic tests. Finally, a generic Standard Operating Procedure (SOP) was compiled to be used for the integrated chemagen-Perkin Elmer extraction platform.
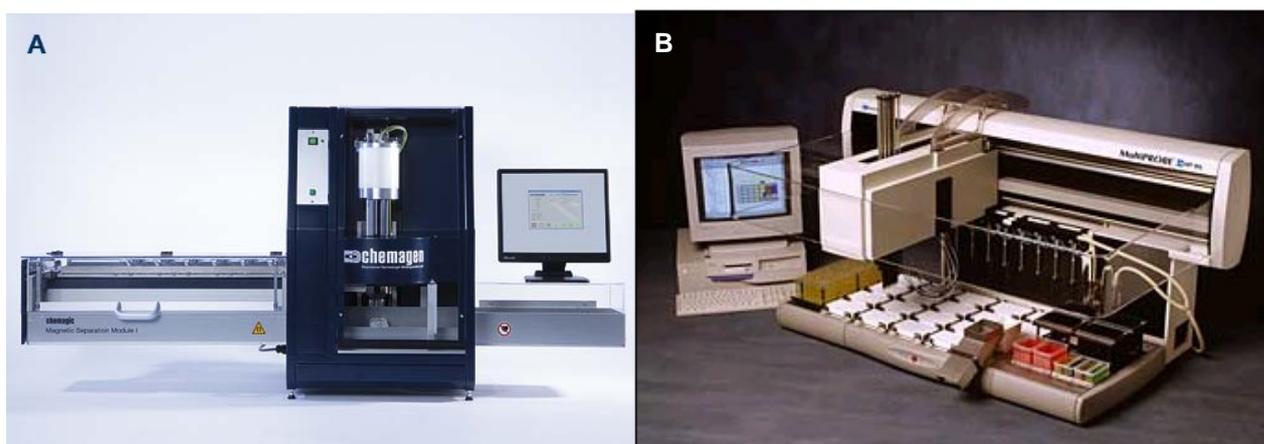

Figure 1: A: chemagic Magnetic Separation Module I. B: Perkin Elmer Multiprobe II liquid handler.

2. Platform Description and Principle

2.1 Extraction chemistry

The chemagen chemistry for DNA extraction from whole blood is based on the use of paramagnetic beads. In the first step, the lysis of the white and red blood cells is performed, in the presence of protease for protein degradation. The isolation of the DNA is achieved through its capturing by magnetic polyvinyl alcohol beads (M-PVA Magnetic Beads). The coating of these beads is binding the DNA with a high specificity. When applying an electromagnetic field, these beads, together with the bound DNA, are attracted to the magnetized metal rods, which can then transfer the DNA from one washing buffer to another. After deactivation of the electromagnet, at the end of each transfer step, the rods start to rotate leading to an efficient and homogeneous resuspension of the particles (Figure 2). In the final step the beads are transferred into elution buffer, which inactivates the interaction between the beads and the DNA. Afterwards the magnetic beads are removed leaving the isolated DNA in suspension.
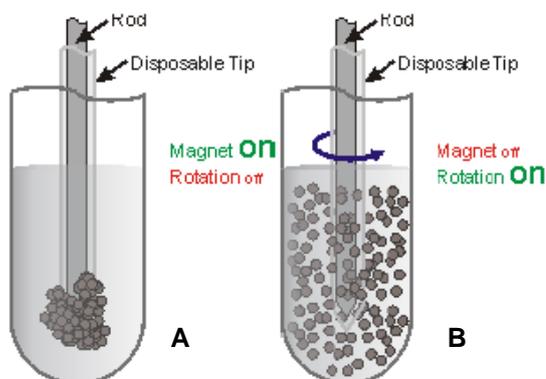

Figure 2: Principle of transferring and resuspending the paramagnetic beads. A: The electromagnet is activated causing an electromagnetic field, which magnetizes the metal rod. This rod is covered by a disposable tip to prevent between-run contamination of the rod. In this situation the beads, together with the DNA, are attracted by the rod, and can be transferred. B: After the transfer the electromagnet is deactivated and the rotation of the rod is switched on causing the beads to be homogeneously resuspended in the buffer solution.

2.2 chemagic Magnetic Separation Module I

The chemagic MSM I from chemagen is a benchtop extraction robot. The instrument can be used both for DNA extractions starting from small (< 1.5 ml) and large (> 1.5 ml) volumes of blood, where only the rod head needs to be changed depending on the volume. For small volumes the 96 rod head is applied which can thus simultaneously extract 96 samples, whereas the 12 rod head is used for large volumes of up to 10 ml (Figure 3). The extraction of small blood volumes is out of the scope of this document.
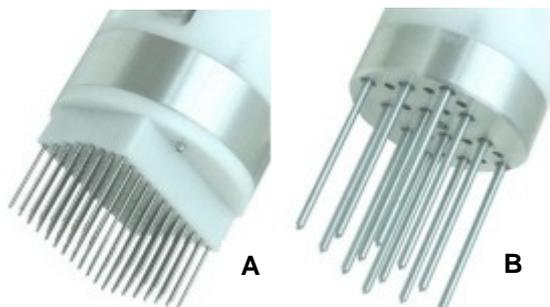

Figure 3: A: 96 rod head to be used for small blood volumes (< 1.5 ml), allowing 96 samples to be extracted simultaneously. B: 12 rod head for extraction of blood volumes up to 10 ml, allowing 12 samples to be extracted simultaneously.

2.3 Perkin Elmer Multiprobe II Expanded Liquid Handling robot

The Perkin Elmer MPII Ex is an 8-tip automated liquid handling robot, equipped with two 6-way valves offering the ability to install up to 11 different reagent containers, including the system liquid. All tips can be programmed independently from each other in any x-y-z position on the deck. In our case, the deck offers 32 microplate tiles for sample racks and a gripper, which is used to transfer racks to different positions. The setup in our lab was suitable for up to three consecutive extraction runs without a manual intervention, but the scope of this project was limited to one entirely automated extraction run. Other adjustments, e.g. two consecutive runs followed by automatic DNA normalization on deck, are possible as well. A barcode reader is included for traceability reasons, however, this reader was not used for this project.

2.4 The chemagen – Perkin Elmer integration

For this specific project the two systems were integrated as an automated DNA extraction platform for large volumes of blood. Both software interfaces were set up in a way that they would interact as streamlined as possible. The instruments are coupled in a way that the chemagen array can be loaded with tube racks by the Perkin Elmer robot, which is equipped with a gripper for this purpose. In this way, all tubes can automatically be filled with the proper reagents by the Perkin Elmer robot and afterwards placed on the chemagen array in the correct order for DNA extraction. A picture of the integrated setup is given in Figure 4.

Figure 4: Integration of the chemagic MSM I extraction robot (B) with the Perkin Elmer MPII liquid handler (A). Note that the 12 rod head (1) is situated just above the electromagnetic ring (2). After the tubes in the racks (3) have been filled by the 8-tip pipetting system (4), the racks are picked up by the gripper (5) and placed on the chemagen array (6). This array can move along the x-axis in order to place the correct tubes under the magnetic rod head. The rods can move along the y-axis and thus descend into the fluids in the tubes to attract the beads together with the DNA or to rotate and thus homogenize the solution. The entire process is controlled by one computer (7) with both software packages installed.

3. Extraction Procedure

3.1 Standard DNA extraction

The Perkin Elmer MPII deck is built up in a way that racks containing 12 tubes (50 ml or 15 ml) or 96 well plates can be placed on the different microplate tiles. All necessary racks with empty tubes need to be placed on the corresponding tiles, together with one rack containing the blood samples. In addition a container with beads solution and several tubes with protease on a cooling block are put at the appropriate position on the deck. A typical deck lay-out for the standard DNA extraction procedure is schematically represented in figure 5, as it is given by the Perkin Elmer software. All other reagents are directly linked to the system and controlled by two 6-way valves.

After starting up both software packages – chemagen and Perkin Elmer – the remaining steps of the extraction procedure are performed automatically. All necessary reagents are part of the chemagic DNA blood Kit special, art. No. 715.
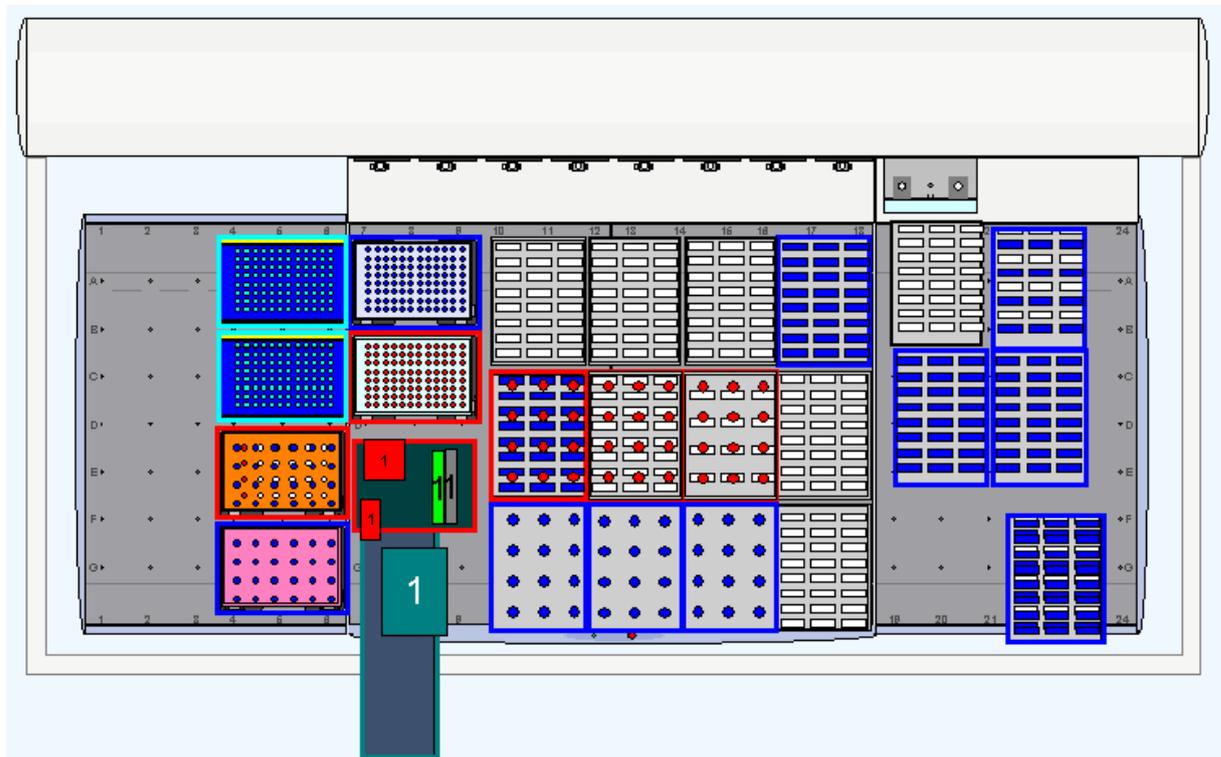

Figure 5: Schematic representation of the PE MPII deck lay-out for a typical DNA extraction. The deck is divided into microplate tiles where either tube racks or 96 well plates can be placed on.

A scheme of the entire procedure is drawn in figure 6. In the first step, 500 µl of elution buffer is dispensed in 15 ml tubes, in which the DNA will be eluted at the end of the extraction procedure. Then protease is added to the blood samples to eliminate all proteins. In addition 9 ml of Lysis buffer is added and the blood samples are placed on the chemagen array. Plastic tips are placed over the metal rods from the chemagen extraction robot to avoid cross-contamination between runs. These rods descend into the blood samples and start rotating to improve lysis of the white blood cells. As soon as the chemagen robot begins the lysis, the Perkin Elmer robot starts a timer for the duration of the lysis (30 minutes). Meanwhile it starts adding the different washing buffers to the empty tubes on the deck.

After lysis of the blood samples, the buffer racks are placed on the chemagen array. To prevent sedimentation of the paramagnetic beads, an extra mixing stap was inserted in the protocol.

Afterwards, the paramagnetic beads solution is added to the lysed blood samples together with a Binding Buffer, which enhances the binding of the DNA to the M-PVA beads. This is the point where the chemagen extraction procedure begins.

For the actual DNA extraction, the DNA – released from the lysed white blood cells – is bound to the M-PVA beads and then subsequently transferred from one washing buffer to another by use of the magnetic rods. Each washing step includes a homogenization. There are four serial washing buffers with different ethanol concentrations. In the final step the DNA is released from the beads in the elution buffer. The beads are then retracted by the magnetic rods and discarded.

It was observed that sometimes there are still traces from the beads present in the final eluates. Therefore, the samples are transferred to a 96 well block on a magnetic stand and left there for 15 minutes. This allows all remnants of the beads to be attracted by the magnets on one side of the wells so that the DNA can easily be transferred to the final sample tubes.
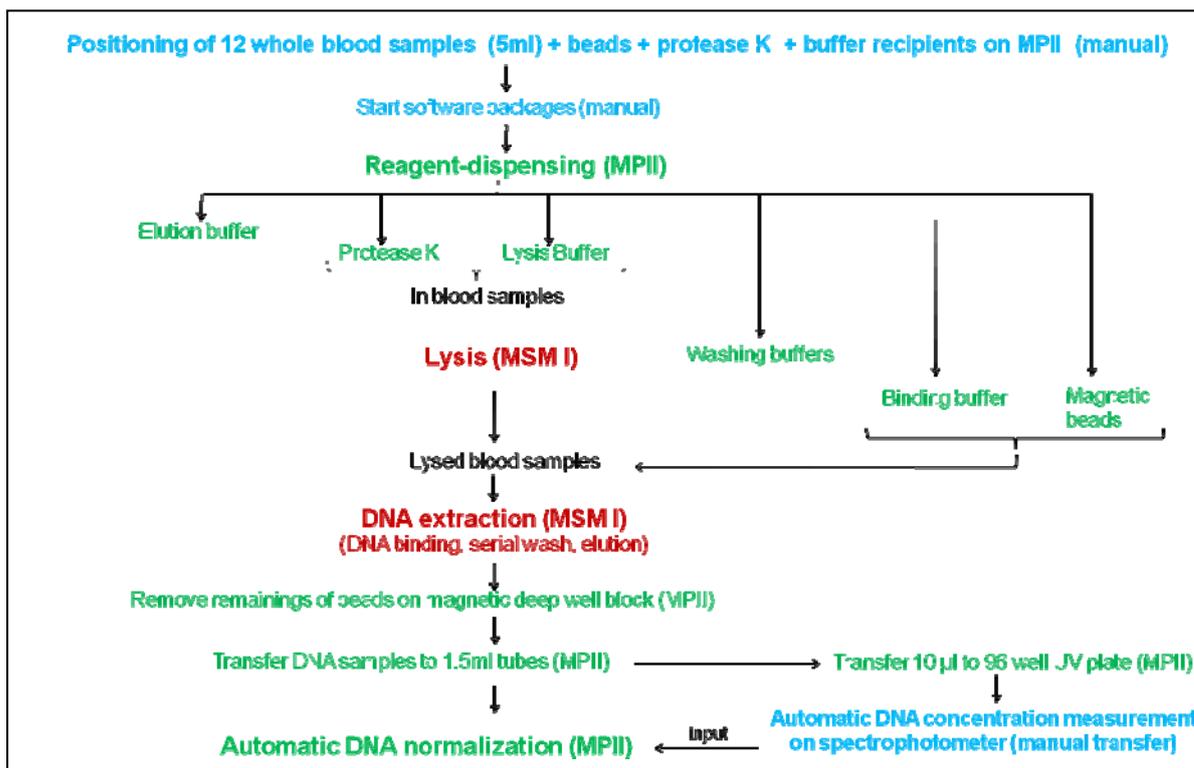

Figure 6: Schematic representation of the extraction procedure performed by the chemagen – Perkin Elmer integrated system. Manual interventions are indicated in blue, whereas the steps performed by the Perkin Elmer and chemagen robot are indicated in green and red, respectively.

3.2 DNA concentration measurement

In a diagnostic setting it is desirable to measure the DNA concentration immediately after the extraction in order to normalize all samples to a fixed concentration. In our laboratory a Victor[3] spectrophotometer (Perkin Elmer) is used for these measurements. The standard protocol requires 10 µl of DNA and 190 µl of water to be dispensed into a 96 well flat bottom UV plate (Costar Art.No 3635). This step can be automatically performed right after the standard extraction procedure (section 3.1). It would even be possible in an adjusted setup to place the Victor[3] on the Perkin Elmer MPII deck as well. This allows the gripper to load the UV plate automatically into the spectrophotometer for DNA concentration measurement and to put it back on the deck for further normalization (section 3.3). However, this configuration was not tested in this validation project.

3.3 DNA normalization

After the DNA concentration measurement a MS Excel macro file can be started to create a CSV file that can be used by the PE MPII software to start the automatic DNA concentration adjustments procedure. Based on the calculations of the macro, a calculated volume of TE buffer will be added to the DNA samples of which the concentration is too high. It is possible to fully automate the entire procedure, starting with the DNA extraction of whole blood samples and resulting in normalized DNA samples at a fixed concentration. Again, this is out of the scope of this validation.

4. Validation Setup and Results

4.1 Samples

For the purpose of this validation project 182 anonymized blood samples were extracted according to the standard DNA extraction procedure (section 3.1) on the chemagen – Perkin Elmer platform. A fixed volume of blood was used, i.e. 5 ml, and only EDTA was allowed as an anticoagulant. The blood samples had to be less than two weeks old when stored at room temperature or 4°C and no samples from patients with blood-related diseases, like for instance leukemia, were included in the study.

4.2 Validation parameters

For this study a number of validation parameters were selected, which were thought to be essential for an automated DNA extraction platform. In the future, some other parameters – thought to be less critical – can always be included in the validation, since this is supposed to be a continuous dynamic process.

4.2.1 Quality

As a standard quality control for the DNA it was decided to perform a multiplex drop-out PCR on all extracted samples, previously described by Van Dongen et al., 2003. The PCR products were loaded on a 2% agarose gel for electrophoresis together with a size marker (SmartLadder for Small Fragments, EuroGentec). When the DNA has a good quality, this PCR will result in five bands, i.e. at 100, 200, 300, 400 and 600 bp (Figure 7). Since the smallest PCR products (100 bp) bind less ethidium bromide, they are expected to give less bright bands after electrophoresis. Thus, poor quality of the DNA is indicated by weaker bands for the largest PCR products, or if some of the expected bands are completely missing.
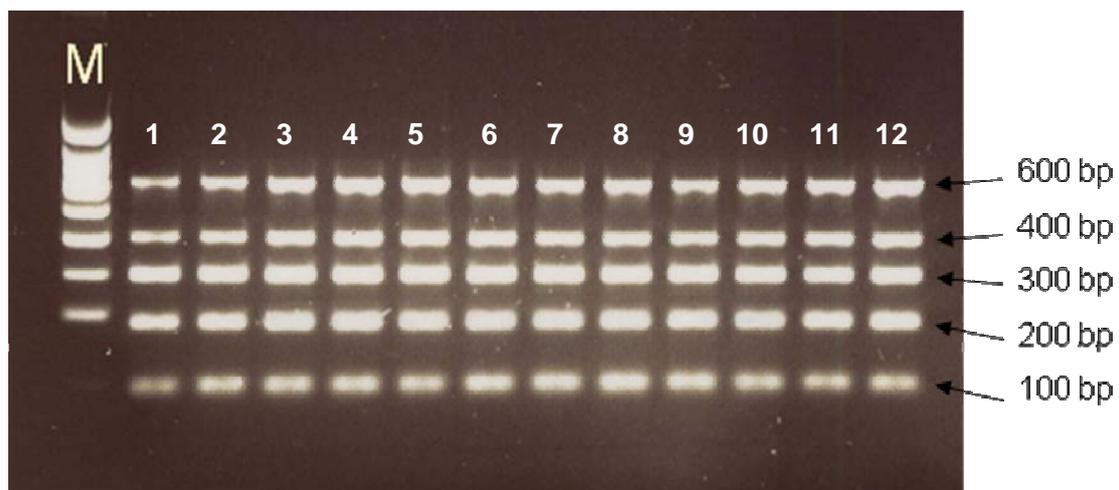

Figure 7: The multiplex drop-out PCR (Van Dongen et al., 2003) as a DNA quality test. Data are shown for 12 random DNA samples extracted with the chemagen – Perkin Elmer platform and normalized to a concentration of 50 ng/µl. The size marker (M) is shown on the left.

As can be seen in Figure 7 all expected PCR fragments are visible. However, somehow weaker 100 bp and 600 bp bands are observed for sample 1. This effect was seen in only 3% (6/180) of the samples, moreover not a single sample showed less than five bands, indicating that DNA extraction with the chemagen – Perkin Elmer platform results in high quality DNA, suitable for good performance in a multiplex PCR.

4.2.2   Degradation

DNA degradation is known to cause significant problems for some diagnostic tests, for instance Southern Blotting analysis. Therefore we selected a pool of 30 DNA samples, randomly distributed over the entire evaluation period, and 500 ng of these samples was loaded directly on a 0.8% agarose gel. If no degradation had occurred, we would expect a single high molecular weight band in the compression zone, as shown in Figure 8.
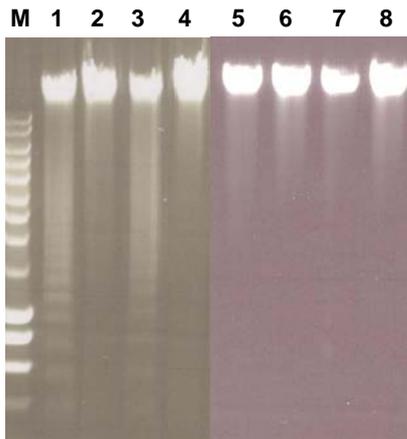

Figure 8: 500 ng of 8 randomly selected DNA samples, extracted with the chemagen-Perkin Elmer platform, and loaded on a 0.8% agarose gel for electrophoresis. The size marker (M) has an upper band of 10 kb.

The results for this degradation test were quite variable with some samples having a single thick high molecular weight band above 10 kb (figure 8 5-8), while other samples showed a strong smear or fragmentation below 10 kb (figure 8 1-4).

We investigated the age of the blood samples before extraction for some of the samples and the results of the degradation test are shown in figure 8. We found out that the age of samples 1 to 4 before extraction was respectively 16, 2, 6 and 0 days. These results are confirmed by the degradation profile where the degradation is the strongest for sample 1 (16 days old before extraction) and the lowest for sample 2 and 4 (2 and 0 days old before extraction).

Our findings indicate that there is generally no or very little (very light smear) DNA degradation for the samples extracted with the chemagen – Perkin Elmer platform. However, the storage time of the blood samples before extraction appears to be critical. When samples are stored at room temperature for over 4 days, fragmentation becomes visible on agarose. This fragmentation is believed to be caused by gradual cell apoptosis of the white blood cells. Therefore it is strongly suggested to use blood samples as fresh as possible for extraction, or to store them at 4°C instead of at room temperature.

4.2.3   Carry-over contamination

Since DNA extraction is the very first step in all molecular diagnostic tests, it is extremely important that there is no cross-contamination at this stage between samples. This could occur for example if blood droplets from one sample are dropped in a different tube. The chemagen MSM I counters this problem by moving a tip drop cover under the rods every time the array changes its position.

However, to conclusively investigate this we performed four extraction runs, each time on 12 samples, i.e. six blood samples alternated with six blank samples (1x PBS), in a checkerboard pattern (Figure 9 A).
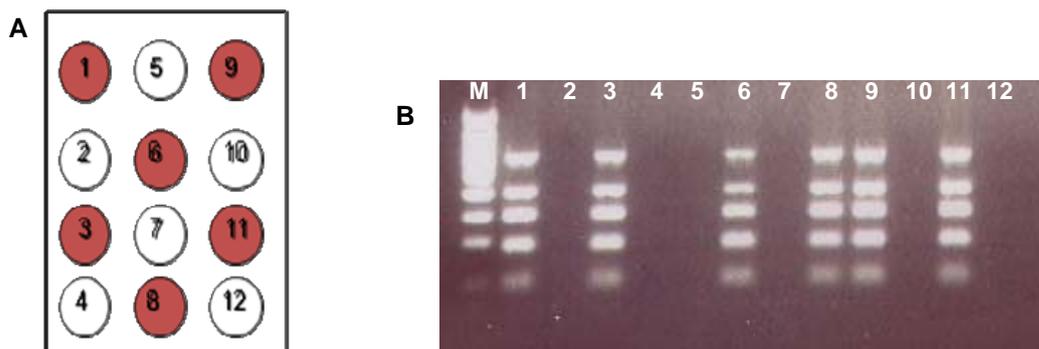
Figure 9: A: Set up for contamination test. Red circles represent blood samples, white circles represent blank samples (1x PBS). If the first run had a blood sample in position 1, the second run would have a blank sample at that position to make sure that all possible combinations were assessed. B: Multiplex drop-out PCR on one of the runs. The size marker (M) is shown on the left. Samples 1, 3, 6, 8, 9 and 11 are extracted blood samples, while 2, 4, 5, 7, 10 and 12 are extracted 1x PBS samples.

A first indication of possible contamination could be retrieved from the DNA concentration measurement immediately after the extraction. If no contamination had occurred values very close to zero for the blank samples would be expected. However, to prove that there was absolutely no carry-over between the samples in a run, a multiplex drop-out PCR, discussed in section x, was performed on both blood and blank samples, where obviously no bands were expected for the blanks.

The results showed that no bands were observed for any of the blank samples. This proves that no measurable carry-over contamination had occurred in any of the runs. An example of such a run is given in figure 9 B.

4.2.4   Concentration

Measurement of the DNA concentration with a fixed elution volume of 500 µl was required for the calculation of the yield. As discussed already in section 3.2, the concentration was standardly measured immediately after each extraction run by the Victor$^3_{TM}$ spectrophotometer. However, for comparison matters, the same measurement was performed by the Nanodrop^{TM} 1000, which generates some additional information about the purity of the DNA as well (section 4.2.6).
A distribution plot of the DNA concentration ranges is given in figure 10.

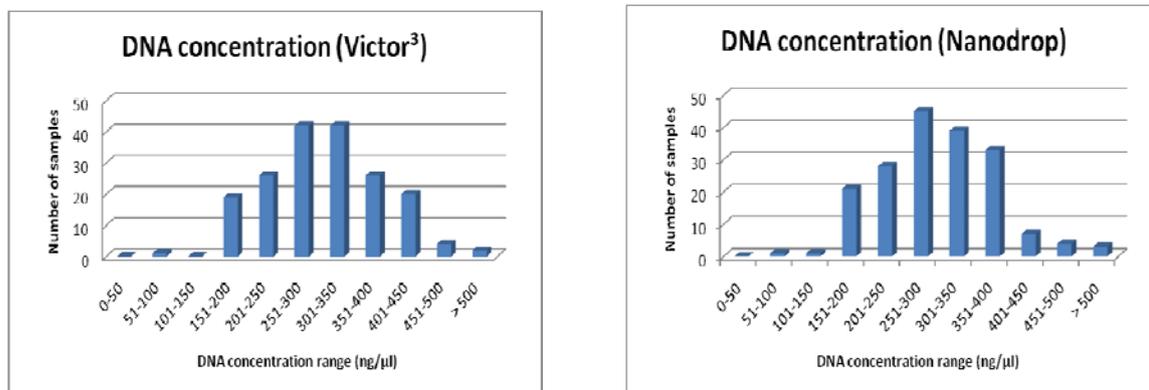Figure 10: The distribution of the DNA concentration as measured by the Victor$^3_{TM}$ (left) and by the Nanodrop^{TM} 1000 (right) for n=182.

Figure 10 shows that the plots are not only very similar when compared between the two spectrophotometers, but they follow a normal distribution as well with very few sample numbers for the lowest and the highest concentrations and a maximum in the middle around 300 ng/µl.

Another observation is that the overall DNA concentration is quite variable amongst the samples, despite the fact that all of them were extracted out of a fixed volume of 5 ml whole blood. This might be related to the variability in WBC count between samples, which is further investigated in section 4.2.5.

Figure 11 summarizes the basic statistical values for the concentration data measured by the Victor³$_{TM}$ and the Nanodrop^{TM} 1000.

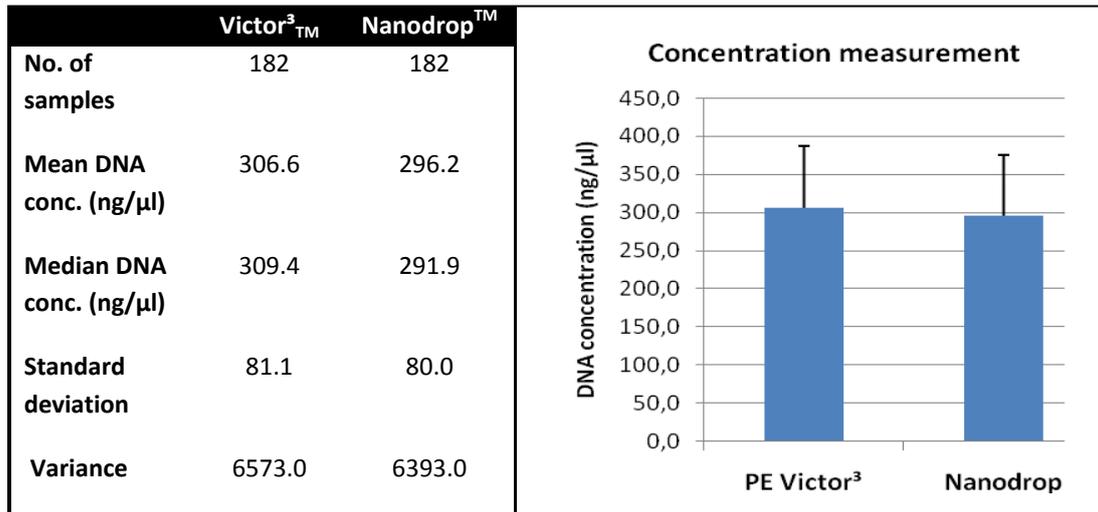|	Victor³$_{TM}$	Nanodrop^{TM}
No. of samples	182	182
Mean DNA conc. (ng/µl)	306.6	296.2
Median DNA conc. (ng/µl)	309.4	291.9
Standard deviation	81.1	80.0
Variance	6573.0	6393.0

Figure 11: Concentration measurement data.

A two-tailed t-test with equal variances indicated that there was no significant difference between concentration measurement on both systems (p > 0.21).

4.2.5 Yield

Following our standard protocol, the extracted samples are eluted in a fixed volume of 500 µl of TE buffer. This means that the total yield per sample can be simply calculated based on the concentration and thus follows the same distribution as for the DNA concentration (section 4.2.4).

The overall DNA yield is depending on the blood sample volume, the WBC count, the efficiency of the extraction method and presumably some other unknown sample-related factors, causing the variable distribution as we have seen it before (section x).

Since the blood sample volume was constant at 5 ml, it was decided to determine the number of white blood cells for all 182 extracted samples using the Z1 Coulter® Particle Counter (Beckman). This allowed us to compare the yield in relation to the WBC count, as represented on the scatter plots in figure 12.

White blood cells are the source of the extracted DNA and therefore the relation was expected to be linear if the extraction method would have a 100% efficiency.

Network for test development harmonization, validation and standardization of services
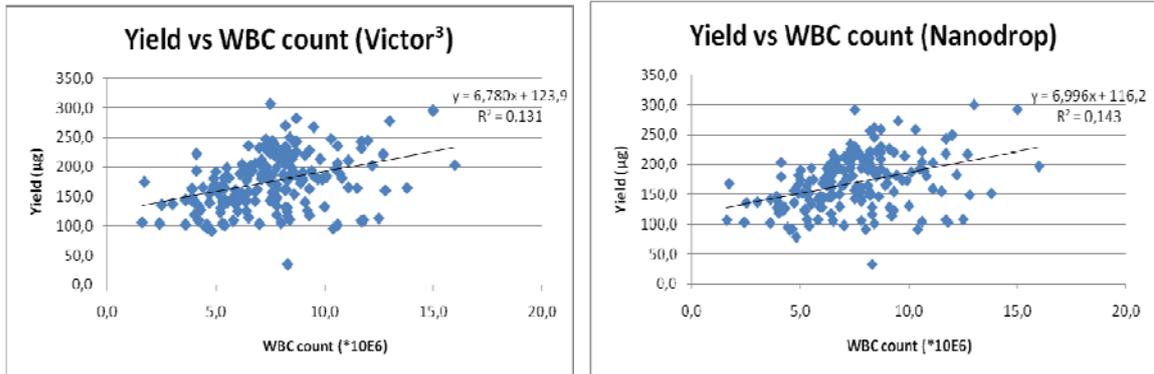
EuroGentest

Figure 12: Scatter plot and linear regression of the yield, expressed as µg DNA per million of white blood cells. The amount of DNA in each sample was measured both with the Nanodrop[TM] 1000 (left) and the Victor[3][TM] (right).

As can be derived from the plots, the majority of the samples have a yield between 100 and 250 µg with an average of 172 µg (SD=46). It should also be noticed that there was only one sample (0.5%) with a yield of less than 50 µg. This means that DNA extraction using the chemagen – Perkin Elmer platform has a success rate of 99.5% for use in molecular diagnostics in general.

Despite the slightly positive trend observed on the plots, there is no significant relation between yield and WBC count with R^2 values of 0.13 (Victor[3]) and 0.14 (Nanodrop), respectively. This means that the observed variability in DNA concentration and yield is related to either some other sample-specific factors or the variable efficiency of the extraction method. The latter can be estimated by investigating the repeatability and reproducibility.

4.2.5.1 Repeatability

Repeatability can be defined as the closeness of agreement between replicates of the same sample in the same run. Therefore the same blood sample was extracted three times within the same extraction run. This was done for four test patients. Since the amount of white blood cells for a given blood sample is constant, one would expect a similar yield for the replicates of that sample, if the extraction method is consistently efficient. Data are given in figure 13.
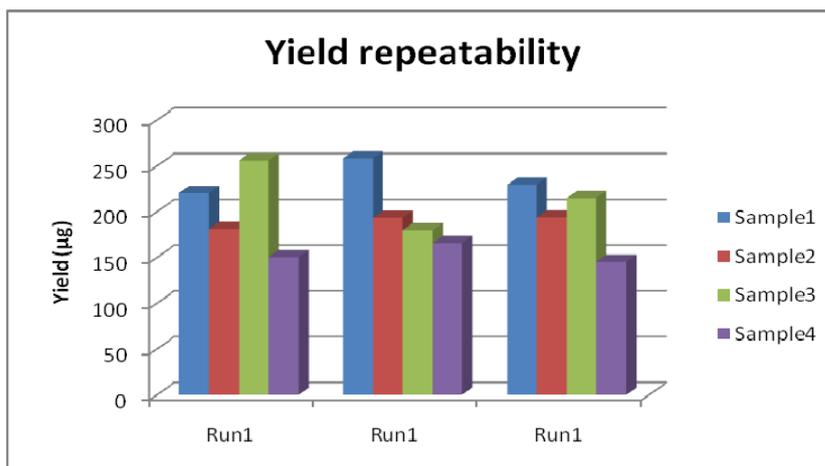

Figure 13: Yield data of four blood samples, extracted three times in the same run.

With exception of sample 3, all blood samples result in a very similar yield over the three replicates, suggesting a highly consistent efficiency within the same run.

4.2.5.2 Reproducibility

Reproducibility can be defined as the closeness of agreement between replicates of the same sample in different runs, under different circumstances. Therefore the same blood sample was extracted in three different runs on three different days by three different operators. Again, this experiment was performed with blood samples from four test patients. Data are given in figure 14.
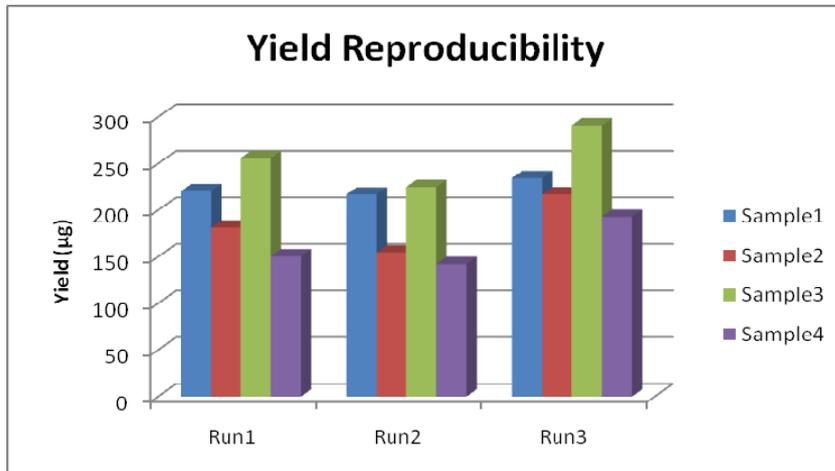

Figure 14: Yield data of four blood samples, extracted in three different runs.

Again, the yield data are similar for all samples. Interestingly, for each sample the yield is the highest in run 3, which was performed on the third day after collection of the blood sample. This indicates a possible effect in efficiency of the extraction, related to, perhaps, the age of the blood samples, or the efficiency of the resuspension of the paramagnetic beads. These are just examples of the many possible sample-specific factors, causing the variability in overall yield.

4.2.5.3 Blood volume

Unlike our assessment study set up, a routine diagnostic laboratory is very likely to receive patient blood samples with variable volumes, which might have an effect on the resulting yield. To evaluate this parameter, three test patient blood samples were extracted at different starting volumes, ranging from 1 to 10 ml. After the extraction, the DNA concentration was measured and the yield was determined. Data are shown in figure 15.
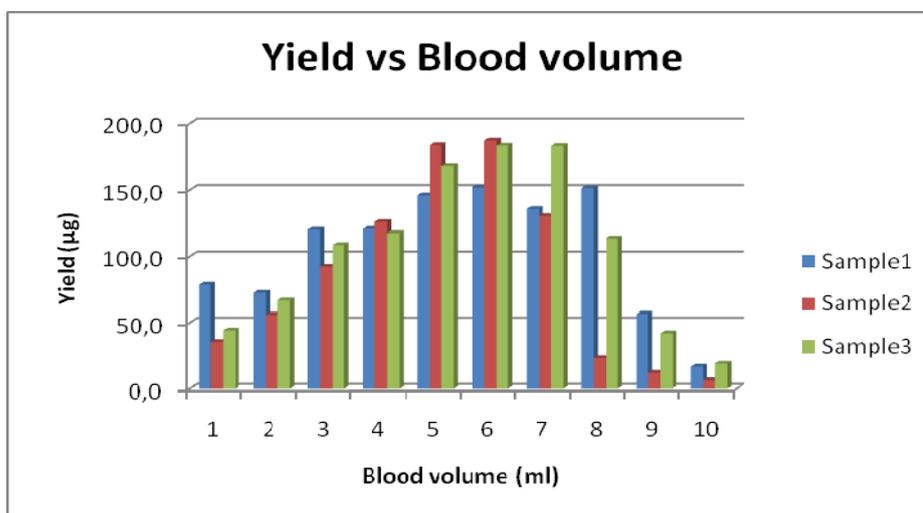

Figure 15: Effect of blood volume on yield.

Network for test development harmonization, validation and standardization of services

EuroGentest

The results show a Gaussian distribution of the yield when ranging the blood volume from 1 to 10 ml. The maximum yield was reached with a start volume of 5 or 6 ml, whereas an acceptable amount of DNA is extracted with start volumes ranging from 3 to 7 ml. Lower volumes result in lower yields due to a reduced number of white blood cells. Higher volumes are not advised to be run using this specific protocol, since it has been developed for blood volumes between 3 and 7 ml and thus will not result in good yields for blood volumes over 7 ml. For these volumes other extraction protocols are available from chemagen.

4.2.5.4 Extreme storage conditions

In some cases it is possible that blood samples reach the molecular diagnostic laboratory after quite bad storage conditions, which might have an influence on the DNA extraction. We have briefly evaluated this for both very old and frozen blood samples.

A small number of very old blood samples, i.e. stored at 4°C for six months, and frozen blood samples, i.e. stored at -20°C over night, were extracted using the chemagen – Perkin Elmer extraction platform. Data concerning quality, degradation, yield and purity were compared to the data from normal blood samples.

Table 1: Summary of tested parameters for extreme blood sample storage conditions.

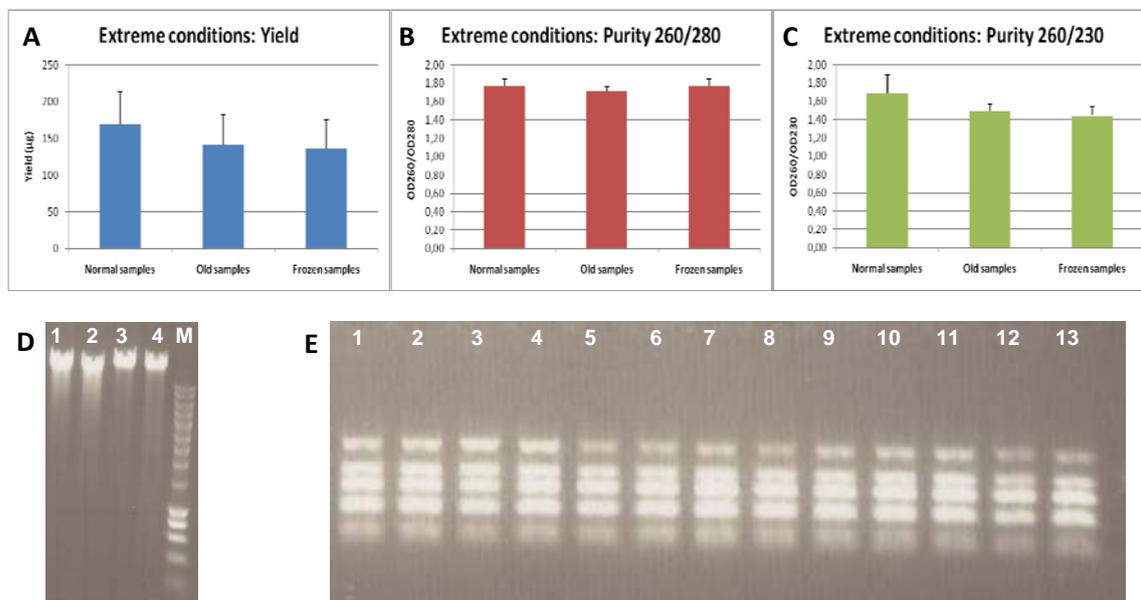| Sample type	DNA yield (µg)	OD260/OD280	OD260/OD230
Normal	168.6 (SD=45.7)	1.78 (SD=0.07)	1.69 (SD=0.19)
Old	141.3 (SD=41.1)	1.72 (SD=0.04)	1.50 (SD=0.07)
Frozen	135.9 (SD=39.7)	1.77 (SD=0.07)	1.45 (SD=0.09)

Figure 16: Comparison of extreme blood sample storage conditions to normal blood samples. Mean results are shown for yield (A), purity estimated by OD260/OD280 ratio (B) and purity estimated by OD260/OD230 ratio(C). D shows the degradation profile for DNA extracted from old blood samples (1-2) and frozen blood samples (3-4) next to the 10kB size marker (M). E is the result of the multiplex drop-out PCR performed on DNA from normal blood samples (1-8), from old blood samples (9-11) and from a frozen blood sample (12).

Generally, the results in figure 16 indicate that the DNA extracted from blood samples that were stored very long or frozen, performs slightly worse than it does for normally stored blood samples.

Yield is clearly lower (figure 16A) and the purity, especially when estimated with the most sensitive method (OD260/OD230), has decreased as well, yet not significantly (figure 16B-C). However, it is not clear which organic component is causing this lower ratio.The higher degree of DNA degradation for old blood samples was already expected based on the results in section 4.2.2 (figure 16D).

Based on these results, we wouldn't recommend using blood samples stored at such extreme conditions, on a regular basis for DNA extraction in a molecular diagnostic laboratory. However, despite the non-optimal results, the DNA still proved to be performing sufficiently well in the multiplex drop-out PCR (figure 16E), illustrating the robustness of the this extraction chemistry.

4.2.6 Purity

For many diagnostic tests it is important that the DNA has a high purity. Nucleic acids, like DNA, absorb UV light with a maximum around 260 nm. Proteins are known to frequently contaminate DNA samples and absorb the UV light at other wavelengths, including 230 nm and 280 nm.

Historically, the purity of a DNA sample was estimated by taking the ratio of the optical density (OD) at 260 nm and 280 nm. Pure nucleic acids are expected to have such a OD260/OD280 around 1.8. Lower ratios will indicate the presence of impurities absorbed at 280 nm, mostly proteins.
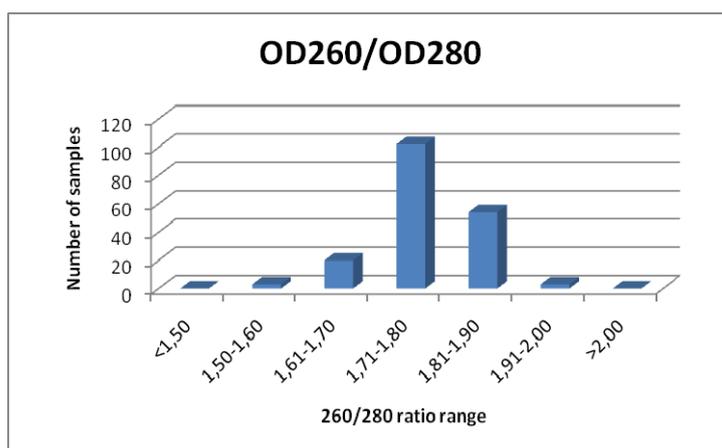

Figure 17: Distribution of the OD260/OD280 ratio ranges for all extracted samples. The absorbance was measured by the Nanodrop[TM] 1000 spectrophotometer.

As can be derived from figure 17, most of the extracted DNA samples have a OD260/OD280 between 1.71 and 1.80. Moreover, 86% (156/182) of the samples is situated around a ratio of 1.8 (± 0.1), and the mean ratio was 1.78 (SD=0.07), suggesting a high purity for the majority of the samples.

However, because only the aromatic side chains of amino acids are absorbed at 280 nm, some proteins with few of these side chains, have a very low absorbance at this wavelength and are thus very difficultly detected.
Since at 230 nm the peptide bonds, as well as many organic components are absorbed, taking the OD260/OD230 is a much more sensitive way to determine protein impurities in a DNA sample. High purity DNA is then expected to have a OD260/OD230 around 2.0.
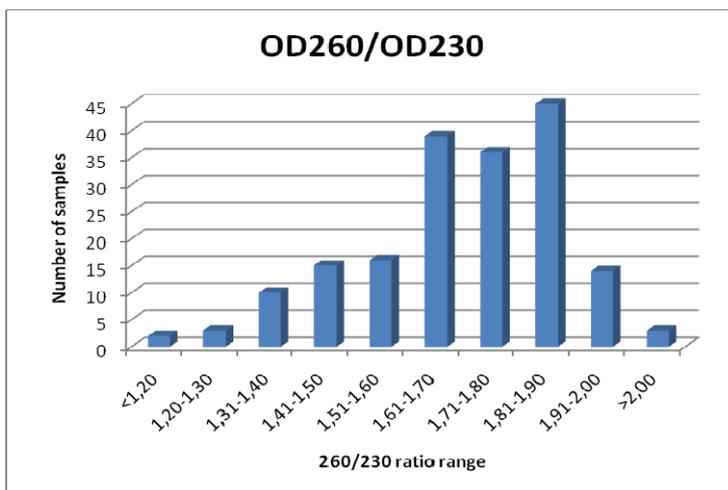
Figure 18: Distribution of the OD260/OD230 ratio ranges for all extracted samples. The absorbance was measured by the Nanodrop™ 1000 spectrophotometer.

Figure 18 shows that the data for the OD260/OD230 are more dispersed. A quarter of the samples (45/182) have a OD260/OD230 between 1.8 and 1.9. Only 10% (18/182) are in the high purity range around 2.0 (± 0.1), with a mean ratio of 1.69 (SD=0.19).

The data suggest that our samples clearly contain some degree of organic impurities. One of the possibilities for this is the EDTA, which is present in the elution buffer and causes absorption at 230 nm. However, based on the fact that 84% (153/182) of the samples gave a 260/230 ratio between 1.5 and 2.1, it is believed that this organic 'contamination' is not critical.
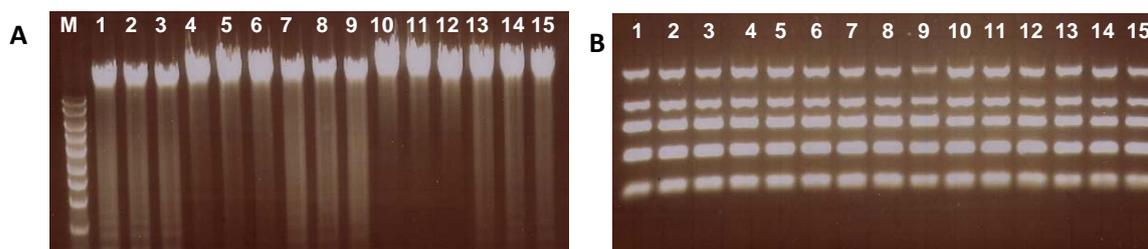
4.2.7 Stability

Most molecular diagnostic laboratories store their DNA samples for very long periods of time, to perform possible additional tests at a later stage. Therefore it is important that the extracted DNA is sufficiently stable to still have a good performance after a certain amount of time.

A stability experiment was set up, to simulate some extreme conditions to which a DNA sample could be subjected. For this purpose, eight DNA samples, randomly selected from the pool of 182 samples, were split up into three aliquots to be stored under different conditions.

The first aliquot of each sample was placed in a freezer at -20°C, which for many laboratories is the standard storage temperature for DNA samples. However, these aliquots were thawed to room temperature and frozen again every working day, to mimic frequent freeze-thaw cycles. Another aliquot was stored in a fridge at 4°C and left there for the entire period to serve as a control. Finally, the third aliquot was put at 37°C, which is the optimal temperature for many enzymes to work and thus to possibly degrade the DNA.

These samples were monthly tested for DNA degradation on a 0.8% agarose gel (section 4.2.2) and for DNA quality by a multiplex drop-out PCR (section 4.2.1). Enough sample volume was provided for each condition to be able to perform this experiment for the period of one year. Figure 19 shows the results at the beginning and at the end of the experiment.

Network for test development harmonization, validation and standardization of services
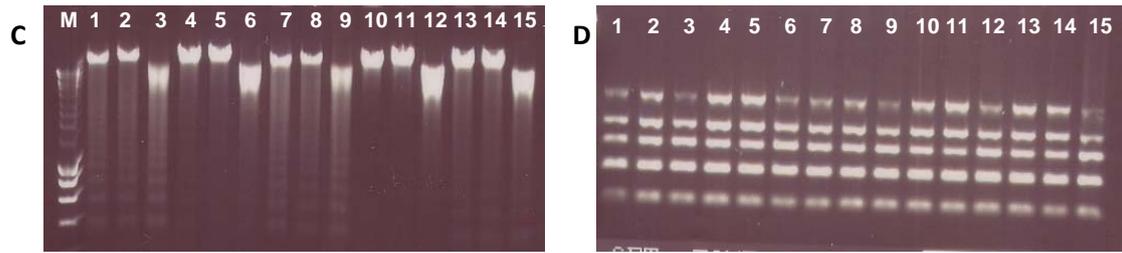
EuroGentest

Figure 19: Stability testing assessed by the DNA degradation test in the beginning (A) and after one year (C), and by the performance in a multiplex PCR (B and D). The same samples (1-15) were used every time and are clustered in groups of three, representing storage at -20°C with freeze-thaw cycles (1, 4, 7, 10 and 13), at 4°C (2, 5, 8, 11 and 14) and at 37°C (3, 6, 9, 12 and 15).

It is very clear that the DNA degradation has the highest impact on the samples that were stored at 37°C. The best results, i.e. the highest molecular weight bands, are generally observed for the samples that were stored constantly at 4°C.

When looking at the performance in the multiplex PCR, one year after extraction all samples are still capable to perform sufficiently well, albeit less good for all samples stored at 37°C and for some at -20°C together with the daily freeze-thaw cycles.

In general, DNA extracted with the chemagen – Perkin Elmer extraction platform is stable enough to perform well up to at least one year after extraction, when stored under appropriate conditions.

4.2.8   Performance

In addition to the assessment of the general DNA quality by the multiplex drop-out PCR (section 4.2.1), it is important for the validation of the extraction platform to evaluate the performance of the extracted DNA in molecular diagnostic tests as well. For this purpose, a random selection of the extracted samples was anonymously included in a series of different types of diagnostic tests, which are summarized in table 2.

Table 2: Overview of the molecular diseases that have been tested with a selection of the extracted DNA samples.

Test subject	Test description
Hemochromatosis	PCR + mutation detection with target-specific probes
Factor V deficiency	PCR + mutation detection with target-specific probes
Apolipoprotein E	PCR + mutation detection with target-specific probes
Uniparental Disomy	PCR + detection of polymorphic marker regions
Y chromosome microdeletions	Multiplex PCR
Cystic Fibrosis	Multiplex PCR + reversed dot blot principle
Huntington Disease	PCR + trinucleotide repeat sizing (capillary electrophoresis)
Spinocerebellar Ataxia	PCR + trinucleotide repeat sizing (capillary electrophoresis)
Von Hippel-Lindau Syndrome	Multiplex Ligation-dependent Probe Amplification (MLPA)
Hereditary Breast Cancer	PCR + DHPLC + sequencing + MLPA

Network for test development harmonization, validation and standardization of services

Charcot-Marie-Tooth Disease	Multiplex Ligation-dependent Probe Amplification (MLPA)
Hereditary Non-Polyposis Colon Cancer	Multiplex PCR + microsatellite instability testing
Kennedy Disease	PCR + trinucleotide repeat sizing (capillary electrophoresis)
Duchenne Muscular Dystrophy	Multiplex Ligation-dependent Probe Amplification (MLPA)
Fragile X Mental Retardation	PCR + capillary electrophoresis + Southern Blotting
Hereditary deafness (COCH + Cx26)	PCR + restriction digestion / allele-specific PCR
Steinert Disease	PCR + capillary electrophoresis + Southern Blotting
Noonan Syndrome	PCR + DHPLC + sequencing
Spastic Paraplegia	PCR + DHPLC + sequencing

Apart from the diagnostic outcome, all selected samples gave good, analyzable and diagnostically interpretable results (data not shown). This indicates that the DNA extracted by the chemagen – Perkin Elmer platform, is generally suitable for use in molecular diagnostic testing.

5. Problems and optimization

5.1 Ethanol

For some disease-specific genetic tests, DNA samples extracted in our laboratory with the chemagen – Perkin Elmer platform, are sent to other laboratories to perform the actual test. Prior to the validation study, and as part of the initial evaluation, we have seen that some samples appeared to work poorly or not at all for certain tests, whereas the same test worked perfectly fine on DNA extracted with the manual salting-out extraction method. Therefore we started to investigate the DNA samples in more detail by High Performance Liquid Chromatography (HPLC), which is commonly used in clinical chemistry for the detection of organic contaminants. These analyses revealed that the DNA samples extracted with the chemagen – Perkin Elmer platform contained around 10% of ethanol. This massive amount of ethanol could have an influence on many molecular tests, like for instance Southern blotting, where the DNA would literally "swim" out of the wells.

Other laboratories that were known to use this chemistry for their DNA extractions as well were contacted and asked to send a small number of samples for ethanol determination.
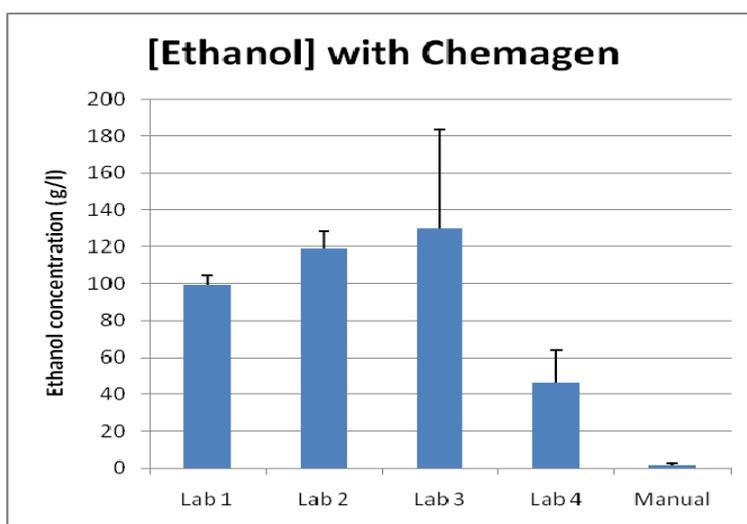

Figure 20: Ethanol concentrations as determined by HPLC for a number of DNA samples extracted by different laboratories using the automated chemagen MSM I extraction robot. Samples that have been manually extracted with the NaCl based salting out method were included as a reference control. Standard deviations are shown for each measurement.

Figure 20 indicates that the problem with the massive amounts of ethanol in the extracted DNA samples is reproducible between different laboratories. The overall mean for the ethanol concentration detected in the DNA samples was 98.6 g/l (SD=37.2) or 10%. For comparison, the mean ethanol concentration in the manually extracted DNA samples was only 1.8 g/l (SD=0.9) or 0.2%.

Despite the rather reproducible measurements for the first three laboratories, it appears that the fourth lab has DNA samples with a significantly lower amount of ethanol. This is probably caused by the fact that the examined samples from this laboratory were much older than the others, causing a large portion of the ethanol to be evaporated.

These data were discussed with chemagen engineers who were keen on finding a solution for this apparent problem. A collaborative optimization process of the automated extraction procedure was set up and the final outcome was to add an extra step to the procedure. This would involve an extra washing step with acetone, followed by a drying step of 15 minutes. This

should be sufficient to eliminate the acetone in the sample, which evaporates much faster than ethanol.

Five DNA samples extracted with this new protocol were subjected to HPLC ethanol determination and compared to five samples extracted with the old protocol.
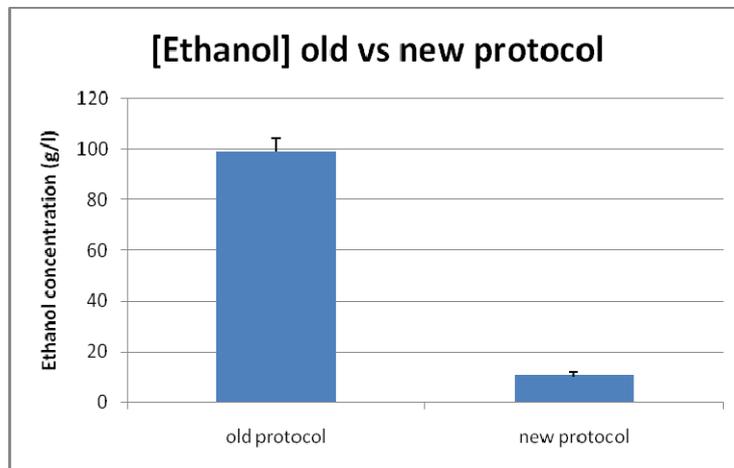

Figure 21: Comparison of the ethanol concentration for DNA samples extracted with the old standard protocol as described in section 3.1 versus the newly optimized protocol with the extra acetone washing step. Standard deviations are shown for both measurements.

As shown in figure 21 the mean ethanol concentration drops from 10% to less than 1%, i.e. a 10-fold decrease, just by adding the acetone washing step to the standard protocol. The samples were sent to one of the external laboratories to be included in a problematic molecular test, which resulted in a significant improvement of the analysis. Therefore it was decided to stick to this adapted protocol for the entire validation study.

5.2 Taqman® SNP Analysis

After the validation study was finished, some DNA samples extracted with the chemagen – Perkin Elmer extraction platform were included in a Taqman® SNP analyis (Applied Biosystems). This analysis appeared to be problematic for this DNA while it performed good for DNA samples extracted with other chemistries.
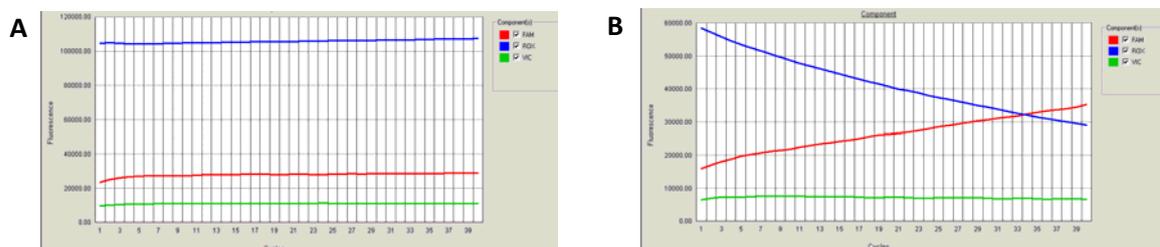

Figure 22: Results from a Taqman® assay. A: The typical profile from a control run. B: The Taqman® profile when analysis is being performed on a chemagen DNA sample. The blue line (ROX) is used for normalization, whereas the red (FAM) and green (VIC) lines represent two alleles that are being examined for certain SNPs.

Figure 22 shows a significant decrease for the ROX signal and an increase for the FAM signal when the Taqman® assay is being performed on a DNA sample extracted with the chemagen chemistry, compared to a control sample, extracted with a different method. These effects disturb the normalization and thus the entire assay.

Since the problem persisted when water was used instead of blood samples for extraction by the chemagen – Perkin Elmer platform, the cause was believed to be inherent to some of the

reagents. Therefore a Taqman® analysis was performed on a sample of the different buffers, resulting in the same pattern as can be seen in figure 22A. However, when the same assay was performed on an aliquot of the M-PVA beads, the problematic picture from figure 22B returned, so the beads were very likely to be causing the interference. Figure 23 visualizes the results for a serial dilution of the beads, which confirmed our hypothesis.
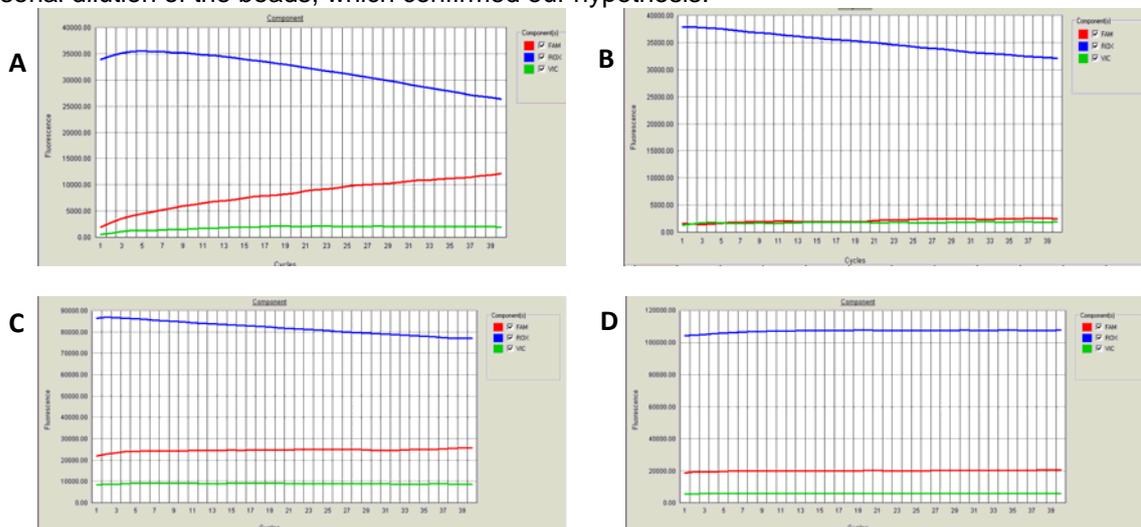

Figure 23: Taqman® results from analysis on a serial dilution of an aliquot of M-PVA beads. Results are shown for a dilution of 1:10 (A), 1:100 (B), 1:1000 (C) and 1:10000 (D).

Further examination revealed that the extracted DNA samples contained significant traces of cerium ions, which are a left-over from the synthesis of the M-PVA beads. These ions are believed to interfere with the Taqman® analysis, causing the pattern shown in figure 22.

The problem was communicated to chemagen and an action plan resulted in a binary solution. First, the purification process in the production of the beads was optimized by the company, leading to more purified beads with less cerium ions. In addition, the elution step has been adapted so that it occurs in a Tris buffer instead of TE. However, EDTA can be added afterwards. So again, this problem has been solved in collaboration with the manufacturer.

The DNA samples extracted with the new protocol on the chemagen – Perkin Elmer platform appear to show no more deviating signals (data not shown). The adaptations are not expected to have an impact on the general performance of the system.

5.3 Multiprobe II gripper

In an effort to make the entire extraction process as streamlined as possible, we tested the ability of the Perkin Elmer – chemagen platform to perform up to three consecutive extraction runs in a fully automatic setup. The gripper on the Multiprobe II was used in this setup to unload and reload the MSM I array before and after each of the runs. However, unlike its performance when doing only one run, the gripper turned out to be not robust enough yet to perform this task in a reliable way when setting up more than one run consecutively. Therefore it was decided to keep the scope of this project limited to one extraction run at a time.

6. General evaluation

The chemagic Magnetic Separation Module I extraction robot integrated with the Perkin Elmer Multiprobe II liquid handler is an automated nucleic acid extraction system. DNA extraction is in many cases the first key stage for molecular diagnostics. Our study was set up to assess this specific platform as part of a EuroGentest validation project.

Our findings suggest that the DNA extracted using the chemagen – Perkin Elmer system has a good quality, as proven by its performance in a multiplex PCR assay and in several molecular tests.

Concerning DNA degradation we have found that the age of the blood sample before extraction is critical. After four days of blood sample storage at room temperature, the extracted DNA shows fragmentation. This effect becomes worse for longer storage of the blood. Therefore, it is strongly suggested to keep the samples as fresh as possible before the extraction, or at least store them at 4°C.
Despite the non-optimal results when blood samples have been stored for an extremely long period of time or at -20°C, they still appear to perform sufficiently well in a multiplex PCR, indicating the robustness of this extraction method.

The system has proven to be efficient with good results for DNA concentration and yield. Out of 5 ml of whole blood we were able to extract 100 μg or more for the majority of the samples. Moreover, only one sample 'failed' with a yield of less than 50 μg, showing a success rate of 99.5%.

The chemagen – Perkin Elmer platform not only performs well on efficiency, but on consistency as well. This was estimated by evaluating the repeatability and reproducibility, which both have shown to be quite good for blood volumes of 5 ml. In addition, the influence of a varying blood sample volume was assessed, indicating a maximum extraction efficiency for 5 ml to 6 ml blood samples.

Purity of the extracted DNA samples was evaluated as well. Our data suggest that the samples clearly contain some degree of organic impurities. However, based on the fact that 84% of the samples was still within a considerably acceptable range for the 260/280 and the 260/230 ratios, and that the slightly lower values for the 260/230 ratio are possibly caused by the EDTA present in the elution buffer, it is believed that this organic 'contamination' is not critical for a good performance in molecular diagnostic tests.

Assessment of the stability of the extracted DNA showed that after a year of storage, the DNA is still able to perform sufficiently well in a multiplex PCR, even when stored at suboptimal conditions.

As a final quality check DNA samples were included in routine molecular diagnostic tests to evaluate their performance. All results were analyzable and interpretable, indicating that the DNA extracted using the chemagen – Perkin Elmer extraction platform is perfectly suitable for use in molecular diagnostic testing.

Additional strong points of this system are its user friendliness, flexibility (easy change between 12 and 96 rods) for large and small blood volumes, and its adjustability to lab-specific needs.
On the other hand, a couple of weaker points are the fact that it's a very large system, not ready out-of-the-box, it requires a lot of flammable buffers and produces quite some waste.

The authors would like to remind that validation is never finished and thus very dynamic. Examples of future topics to be included in this validation could be multiple extraction runs without manual intervention, implementation of the barcode system, the use of different anticoagulants, tissue extractions, small blood volumes, etcetera.

7. Suggestions for in-house verification

One of the main aims of the entire EuroGentest Network is to assist European genetic laboratories in harmonizing and improving the quality of their validations, while duplication of efforts should be avoided if possible.

Therefore, this validation report is freely available through the EuroGentest website (www.eurogentest.org). The authors suggest that at least the following parameters should be verified within the laboratory again, when installing a chemagen – Perkin Elmer DNA extraction platform.

1. DNA quality

We advise to perform the multiplex drop-out PCR on 24 DNA samples, coming from 4 independent extraction runs.

2. DNA degradation

It is suggested to check possible DNA degradation on 12 DNA samples, coming from 4 independent extraction runs.

3. Yield vs blood volume

Determining the yield for different original blood volumes (see 4.2.5.3) is useful to assess whether the amount of beads being added to the blood samples prior to extraction is optimal.

4. Diagnostic performance

It is advisable to check the diagnostic performance of 5 different DNA samples, extracted by the chemagen – Perkin Elmer platform before deciding to use this DNA as a standard in your assays.

5. Storage conditons

Avoid storing your blood samples for too long or at non-optimal conditions before the actual DNA extraction, in order to get reproducible results.