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Product Development of a KASP Genotyping Assay-Based Commercial Genetic Test for Canine Degenerative Myelopathy

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Abstract

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Over 300 Mendelian diseases of the dog (*Canis lupus familiaris*) have been identified. Over 200 genetic tests for canine disorders are currently available worldwide and the global dog DNA testing market is expected to grow in the future. This bachelor's thesis aimed to establish a framework for the development of animal genetic tests and develop the first commercial, direct-to-consumer, animal-targeted genetic test for Medigoo Oyj Inc., a company specializing in *in vitro* diagnostics (IVD).

A genetic test for degenerative myelopathy (DM), a neurogenerative disease found in dogs associated with single-nucleotide polymorphism (SNP) in superoxide dismutase 1 gene (*SOD1*) (c.118G>A, p.E40K), was developed. The test utilized Competitive Allele-Specific PCR (KASP), a method that can be used to genotype DNA samples in relation to two alleles. A literature review was done on the regulations for animal genetic tests, the genomic database for dogs, clinical signs, genetic basis, epidemiology, and population genetics for DM, and the feasibility of the test with KASP. A sample collection protocol for dog buccal swab samples was created. The KASP assay for detecting the alleles of *SOD1*:c.118G>A was tested with 13 different dog samples. Duplicate samples were collected from each dog. DNA was isolated with two protocols: Protocol 1 (P1) had previously been used with human buccal swab samples at Medigoo and was a shortened version of the original isolation kit's protocol, Protocol 2 (P2). The protocols were compared for differences in DNA yield, purity, and performance in KASP.

Only statistically significant difference was found in concentration in favor of P2. The KASP genotyping assay worked as intended, was repeatable, and genotypes could be determined for all dogs.

Keywords: genetic test, degenerative myelopathy, dog, SNP, KASP, SOD1

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Yli 300 mendelistisesti periytyvää koiran (*Canis lupus familiaris)* sairautta on löydetty. Koirien perinnöllisiin sairauksiin on tällä hetkellä saatavilla yli 200 geenitestiä ja koirien DNA-testausmarkkinoiden ennustetaan kasvavan tulevaisuudessa. Tämän insinöörityön tavoitteena oli luoda puitteet eläingeenitestien kehitykselle ja kehittää ensimmäinen kaupallinen, suoraan kuluttajalle suunnattu eläingeenitesti *in vitro* -diagnostiikkaan (IVD) erikoistuneelle Medigoo Oyj:lle.

Geenitesti kehitettiin degeneratiiviseen myelopatiaan (DM), koirien selkäydinrappeumasairauteen, joka on yhdistetty yhden nukleotidin polymorfismiin (eng. single-nucleotide polymorphism, SNP) superoksididismutaasi 1 -geenissä (*SOD1*) (c.118G>A, p.E40K). Testi pohjautui kilpailevaan alleelispesifiseen PCR (KASP) -menetelmään, jolla voidaan genotyypittää DNA-näytteitä kahden alleelin suhteen. Kirjallisuuskatsauksessa selvitettiin eläingeenitesteihin liittyvä lainsäädäntö, koirien genomitietokanta ja testin toteutettavuus KASP:lla. Lisäksi selvitystä tehtiin DM:n oirekuvasta, geneettisestä perustasta sekä taudin ja tautialleelien esiintyvyydestä eri roduilla. Koirien poskisolunäytteille luotiin näytteenotto-ohjeet. *SOD1*:c.118G>A-alleelien genotyypitykseen tarkoitettua KASP-analyysia testattiin 13 eri koiranäytteellä. Koirista kerättiin rinnakkaisnäytteet ja DNA eristettiin kahdella eri menetelmällä: Protokolla 1:tä (P1) oli aiemmin käytetty ihmisen DNA:n eristykseen poskisolunäytteistä ja se oli lyhennetty versio alkuperäisestä eristyskitin menetelmästä, protokolla 2:sta (P2). Protokollia vertailtiin DNA:n saannon, puhtauden ja KASP-suorituskyvyn suhteen.

Vain pitoisuudessa havaittiin tilastollisesti merkitsevä ero P2:n eduksi. KASP-analyysi toimi tarkoitetulla tavalla, oli toistettavissa ja kaikille koirille voitiin määrittää genotyypit.

Avainsanat: geenitesti, degeneratiivinen myelopatia, koira, KASP, SNP, SOD1

Contents

List of Abbreviations

SOD1:c.118G>A:

Guanine (G) to adenine (A) substitution in the 118th nucleotide of the coding (c) DNA of the *SOD1* gene. Single-nucleotide polymorphism in exon 2 of the superoxide dismutase 1 gene located in chromosome 31 of the dog (*Canis lupus familiaris*) linked to degenerative myelopathy in most studied breeds.

SOD1:c.52A>T:

Adenine (A) to thymine (T) substitution in the 52nd nucleotide of the coding (c) DNA of *SOD1* gene. Single-nucleotide polymorphism in exon 1 of the superoxide dismutase 1 gene located in chromosome 31 of the dog (*Canis lupus familiaris*) linked to degenerative myelopathy in Bernese Mountain Dog.

CanFam 3.1:

Canis lupus Familiaris 3.1. Version 3.1 of the genome assembly, i.e., computationally assembled genome for the dog (*Canis lupus familiaris*).

DM: Degenerative Myelopathy. Adult-onset, progressive, paralyzing, and eventually fatal neurodegenerative disease affecting the spinal cord of dogs.

FAM: Fluorescein Amidite. Synthetic fluorescein dye used in competitive allele-specific PCR genotyping to label alleles.

FRET: Fluorescence Resonant Energy Transfer. An energy transfer phenomenon between two fluorophores. In competitive allele-specific PCR fluorescein-labeled oligos are initially bound to

quencher-labeled oligos, that absorb the emission and prevent any non-specific fluorescent signal before dye-labeled oligo binding to the complementary tail sequence. They are called FRET cassettes.

chr31:g.26540342G>A:
Guanine (G) to Adenine (A) substitution in the 26540342nd nucleotide of the genomic (g) DNA of chromosome 31 (chr31) of the dog (*Canis lupus familiaris*). The same *SOD1*:c.118G>A single-nucleotide polymorphism in exon 2 of the superoxide dismutase 1 gene of the dog, but the location is expressed in genomic DNA.

HEX: Hexachlorofluorescein. Synthetic fluorescein dye used in competitive allele-specific PCR genotyping to label alleles.

KASP: Competitive Allele-Specific Polymerase chain reaction. Fluorescent genotyping method based on PCR, that can be used to detect small genetic variations between homologous DNA sequences: SNPs, insertions, or deletions, and genotype DNA sample in relation to two alleles.

LMN: Lower Motor Neuron. Motor neurons carry electrical impulses from the spinal cord to the skeletal muscles, making them the key components connecting the central nervous system with muscles involved in voluntary movement. LMN degeneration has been witnessed in dogs affected by degenerative myelopathy.

p.E40K: Glutamic acid (E) to lysine (K) substitution in the 40th amino acid residue of a protein (p). Substitution in the SOD1 protein caused by *SOD1*:c.118G>A missense mutation linked to degenerative myelopathy in dogs.

PCR-RFLP:
Polymerase Chain Reaction-Restriction Fragment Length Polymorphism. Method, in which PCR amplification is followed by

restriction enzyme digestion and characterization of PCR products based on different lengths and amounts of DNA fragments that result in unique banding patterns due to sequence-specific restriction enzyme cleavage. Can be used as an alternative SNP genotyping method for example in KASP accuracy validation.

ROX: Carboxy-X-rhodamine. Passive reference dye is used in PCR applications to normalize the fluorescent signals for variations between wells.

rsID: Reference Single-nucleotide polymorphism ID. Unique numerical IDs for specific single-nucleotide polymorphisms in databases.

SNP: Single-Nucleotide Polymorphism. Variation in one specific nucleotide position in DNA caused by a point mutation.

SOD1: Superoxide Dismutase 1. An enzyme in eukaryotic cells' cytosol and mitochondrial intermembrane space protecting cells from oxidative stress. In addition, *SOD1* is the name of the gene encoding the protein of the same name.

SP110: Speckled 110 kDa. Nuclear body protein and a transcription factor. Part of a specific type of nuclear body called promyelocytic leukemia nuclear body, a structure found in nuclei of eukaryotic cells, that affects many cellular processes such as transcription and apoptosis. In addition, *SP110* is the name of the gene encoding the protein of the same name.

UMN: Upper Motor Neuron. Motor neurons carrying electrical impulses between the cerebral cortex and the spinal cord, making them crucial for voluntary movement. UMN degeneration has been witnessed in dogs affected by degenerative myelopathy

1 Introduction

Dogs (*Canis lupus familiaris*) have kept their place as the most common pet worldwide since their domestication from the gray wolf (*Canis lupus*) [1; 2]. Dogs are the first and one of the best examples of domestication, human-induced artificial selection, which has resulted in huge phenotypic variation within the subspecies: There are over 400 recognized dog breeds [1; 3]. Their genetics have been studied widely and to this day over 300 Mendelian diseases with at least one known likely causal variant have been identified [4]. Over 200 genetic tests for inherited canine diseases are currently available worldwide [5]. The global dog DNA testing market revenue was 235 million USD in 2022 and is expected to reach 723 million USD by 2030 [6]. These tests have the potential to help veterinarians understand and identify hereditary diseases and dog breeders to make better breeding decisions that could decrease the prevalence of these disorders, and therefore improve the well-being and life quality of dogs and their respective owners. However, breeding decisions should be thoroughly considered and not based solely on such tests, since it could lead to a loss in genetic diversity which could pose greater harm than the disease itself. [7.] Degenerative myelopathy (DM), a paralyzing and eventually fatal neurogenerative Mendelian disease found in dogs, is associated with single-nucleotide polymorphism (SNP) in superoxide dismutase 1 gene (*SOD1*) (c.118G>A, p.E40K) [8–10]. It is one of the most widespread disease alleles in the dog population [11]. Multiple direct-to-consumer commercial genetic tests for DM have been developed [12]. There is evidence that the introduction of such tests to the market could decrease the prevalence of the disease without risking genetic diversity [13].

Medigoo Oyj Inc. is a Finnish *in vitro* diagnostics (IVD) productizing company that performs genetic tests for consumers. Up to the present Medigoo had developed genetic tests only for humans. However, the company had a desire to expand the market to animal genetic tests. This bachelor's thesis, completed as part of the Biotechnology and Chemical Engineering degree program at Metropolia University of Applied Sciences, aimed to establish a framework for

the development of animal genetic tests for Medigoo by developing their first commercially available genetic test for an animal. Clinical genetic test for dogs was selected as a starting point because dog breed associations had contacted Medigoo and expressed interest in such tests. In addition, dogs are the most popular pets in Finland and globally, thus such tests could reach a broad customer base [14; 15]. To establish the animal genetic testing framework for Medigoo and to develop the genetic test for dogs, several aspects needed to be taken into consideration: the regulatory environment that sets the possible guidelines and limits for animal-targeted genetic tests and their development, the genomic database and genotyping data for dogs, sample collection and DNA isolation protocols for dog DNA samples, genetic underpinnings for the trait being tested, and feasibility of the genetic test in Competitive Allele-Specific PCR (KASP) genotyping platform used at Medigoo's laboratory. The genetic test for DM susceptibility was considered a great candidate as the first clinical genetic test for dogs due to several factors: wide distribution of the disease allele in the dog population, the rather simple genetic basis of the disease, the feasibility of the test with KASP, and a large body of research on DM to support the development of the test [8; 11].

2 Literature Review

2.1 Regulatory Environment

A genetic test can be defined as a laboratory analysis in which the DNA of a blood or tissue sample is analyzed for variations in genes [16]. When the purpose of the use of a genetic test is to determine the genetic susceptibility for a disease, it is seen to have a medical use. Genetic tests with medical use that are meant for humans are classified in the EU as clinical genetic tests which are regulated by regulation on *in vitro* diagnostic (IVD) medical devices (EU 2017/746). Clinical genetic tests targeted to humans are therefore classified as IVD medical devices in the EU. [17; 18.]

Currently, there is no equivalent straightforward classification and harmonized regulatory framework for genetic testing for animals in the European Union or Finland. The Finnish Food Authority is the leading expert in matters considering animal health and welfare in Finland [19]. Their animal welfare department was interviewed, and according to them, clinical genetic tests targeted to animals do not require approval from the authorities in Finland, but law 693/2023 and decree 1165/2023 have sections regulating sample collection from animals and products meant for animals [20; 21]. In addition, for certain types of animal samples, a project authorization is required [22].

2.1.1 Animal Welfare Act (693/2023)

The Animal Welfare Act 693/2023 is a national Finnish law that aims to promote animal welfare, protect animals from harm, and increase the respect and good treatment of animals. It applies to all animals and has several sections that could concern genetic tests for animals. [20.]

Section 15 defines permitted procedures to be done to animals. Subsection 2, paragraph 2 specifies that blood and tissue samples can be taken from animals if it is required by other legislation. [20.]

Section 15, subsection 2, paragraph 5 specifies that procedures causing pain or suffering are allowed if they are necessary to improve the handling of the animals, to ensure the health or safety of animals, to determine the breeding value of the animal, or for any other necessary reason related to the keeping of the animals [20]. This is further elaborated in decree 1165/2023 [21].

Section 17 rules that instruments, equipment, and substances intended for the care and handling of animals must be appropriate for their purpose and must not cause unnecessary pain, suffering, or danger of harm to the animal [20].

2.1.2 Government Decree on Procedures to be Done on Animals and Methods of Artificial Reproduction (1165/2023)

Decree 1165/2023 is a national Finnish government decree that elaborates the permitted procedures to be done to animals mentioned in 693/2023, section 15 [20; 21].

Section 7, subsection 2, paragraph 5 further elaborates on procedures permitted to be done to animals by saying that taking a blood or other tissue sample to monitor or promote animal health is permitted if the sample collection causes only mild or temporary pain [21].

Section 2 defines who is eligible to perform the procedures specified in the decree. Subsection 2, paragraph 2 specifies that a person who is not a veterinarian practitioner is permitted to perform the procedures if a person has sufficient knowledge to perform the procedure in such a way that unnecessary pain or suffering is not inflicted on the animal and the welfare of the animal is not unnecessarily endangered. [21.] This should be taken into consideration in the creation of the sample collection instructions for the test to ensure that consumers have sufficient knowledge to perform the sample collection in a way that does not cause any harm to the dog being tested. In this regard, a buccal swab sample is more infallible than a blood sample.

2.1.3 Project Authorizations

Project authorizations are national Finnish authorizations regarding the use of animals in experiments. They are conceded by the Project Authorization Board. They are required for experiments involving live non-human vertebrate animals and fetal forms of mammals or birds in the last third of their normal development in circumstances when pain, suffering, distress, or lasting harm comparable or higher to the puncture of a needle is inflicted on the animal. [22.] Therefore, development of a canine genetic test that is based on buccal swab samples could be developed without the requirement for project authorization, but blood samples would require the permit.

2.2 Degenerative Myelopathy

Canine degenerative myelopathy (DM) is an adult-onset progressive neurodegenerative disease affecting the spinal cord of dogs. It is characterized by degeneration of myelin and motor neurons which leads to muscle mass loss, muscle weakness, unusual muscle stiffness (spasticity), incoordination (ataxia), partial (-paresis), and complete (-plegia) disability in pelvic (para-) and thoracic limbs, urinary and fecal incontinence, swallowing difficulties, inability to bark, and respiratory dysfunction. The disease is eventually fatal. [10; 23–25.]

2.2.1 Clinical Signs & Disease Progression

The mean age-of-onset of clinical signs of DM varies among different breeds from 8.6 to 10.9 years. While the lowest reported age of onset is 5 years, most affected dogs are at least 8 years of age. The first 6–12 months are characterized by degeneration of upper motor neurons (UMN) in the T3–L3 region of the spinal cord (Figure 1) and paraparesis. [10; 23.] UMNs carry electrical impulses between the cerebral cortex and the spinal cord, making them crucial for voluntary movement [23; 26]. When UMNs are affected, mild, usually asymmetric spastic paresis in pelvic limbs (PL) is witnessed as well as general proprioception (sense of position) ataxia. These are the key clinical signs of DM. [10; 23.]

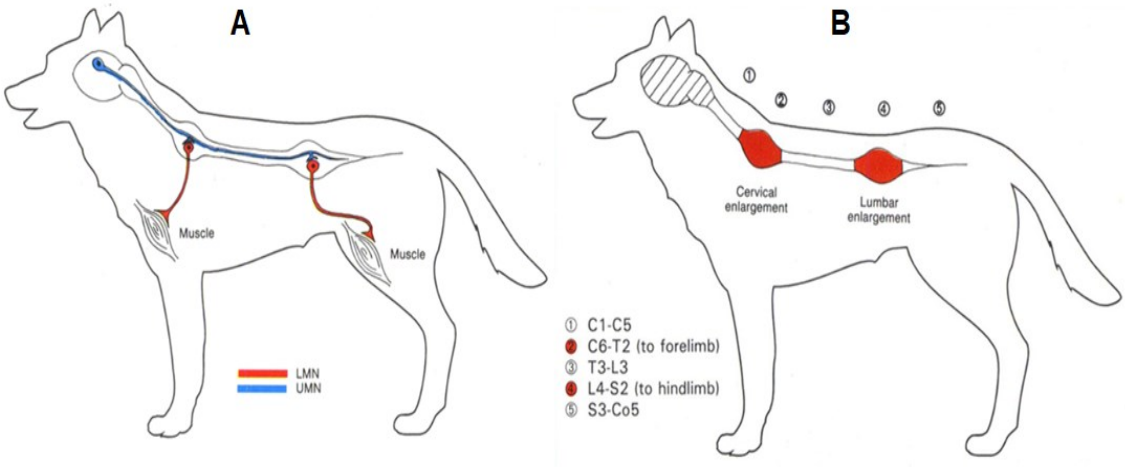

Figure 1. A: Dog neural pathways from the brain down the spinal cord to the muscles of the thoracic and pelvic limbs. Upper motor neurons (UMN) carry information from the cerebral cortex down the spinal cord, while lower motor neurons (LMN) connect the spinal cord to the skeletal muscles, making them both essential neurons for voluntary movement. Both types of motor neurons become affected in DM, explaining why the disease eventually leads to paralysis. B: Dog spinal cord segments. Segment 3: T3–L3 is where the motor neurons and myelin become mostly affected in DM. [10; 23; 26.]

After 8–18 months from the disease onset, the lower motor neurons (LMN) (Figure 1) start to degenerate [10]. LMNs carry electrical impulses from the spinal cord to the skeletal muscles, making them the key components connecting the central nervous system with muscles involved in voluntary movement [23; 26]. During this stage dogs experience paraparesis or paraplegia, which leads to inability to walk as well as mild to moderate muscle mass loss and reduced to absent spinal reflexes in PL. Furthermore, problems holding urine or stool might occur. Dogs are usually euthanized in this stage after losing their walking ability. [10.]

If the dog is kept alive and the disease progresses further, after 14–24 months LMNs continue to degenerate as paraplegia becomes certain. Thoracic limbs become affected as well by becoming weak. Spinal reflexes in PL become absent and they lose a significant amount of muscle mass. Urinary and fecal incontinence becomes common. [10; 23.]

Finally, after 36 months and above the disease progresses to the end stage, in which disability in all 4 limbs (tetraplegia) occurs, swallowing, tongue movements and breathing become difficult, and spinal reflexes in all limbs become absent. Furthermore, reduction or absence in reflexes of cutaneous trunci (thin and wide muscle sheet covering dorsal and lateral walls of the abdomen and thorax) is witnessed. Muscle mass is lost significantly all over the body. Urinary and fecal incontinence continue to arise. [10; 23.] The disease can eventually lead to death by respiratory failure [27].

2.2.2 Genetics & Pathogenesis

For most studied breeds, only one single-nucleotide polymorphism (SNP) in exon 2 of the superoxide dismutase 1 (*SOD1*) gene located in chromosome 31 has been associated with DM as the major causative mutation [8; 23]. The point mutation *SOD1*:c.118G>A (in addition referred to as chr31:g.26540342G>A) leads to guanine-to-adenine substitution in the 118th nucleotide of the coding (c) region, which consequently leads to glutamic acid-to-lysine substitution (p.E40K) in the 40th amino acid residue of the of SOD1 protein, hence making it a missense mutation. A/A homozygosity has been associated with an elevated risk of DM in several breeds. [8; 9; 23; 28.] However, the disease has incomplete penetrance, i.e., not all homozygotes will express the disease and there seems to be variability among different breeds on how much homozygosity predisposes the risk to DM [8; 23]. While *SOD1*:c.118G>A allele has been found widely in multiple breeds and mixed breed dogs, the breed-specific data of A/A homozygosity and risk to DM is limited [11]. However, a strong association between A/A homozygosity and risk of DM has been found in Boxer, Pembroke Welsh Corgi, German Shepherd Dog, Chesapeake Bay Retriever, and Rhodesian Ridgeback [8].

The SOD1 protein is an enzyme (E.C 1.15.1.1) found in the cytosol and mitochondrial intermembrane space of eukaryotic cells. It catalyzes the disproportionation of the oxygen-free radical superoxide (O_2^-) into less reactive

hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) and therefore protects cells from oxidative stress. [29; 30.]

In properly folded wild-type canine SOD1 (WT-SOD1) protein, the E40 is close to K91 and there is a hydrogen bond between these residues. There is no interaction between these residues in the E40K-SOD1 mutant. It has been speculated as the reason why the tertiary structure of E40K-SOD1 is significantly less stable compared to the WT-SOD1. This instability results in a higher number of denatured proteins in the cells. In addition, the observed negative net charge in physiological pH for E40K-SOD1 is lower. These factors could result in the acceleration of amyloid fibril formation and aggregation of the mutant protein. [31.] Aggregation of SOD1 protein in spinal neurons and astrocytes has been witnessed and suggested as an important factor in the pathogenesis of DM. Furthermore, there is evidence of SOD1 aggregates moving from cell to cell and resulting in aggregation of properly folded SOD1 as well. This intercellular propagation of SOD1 aggregates could explain the progressive nature of the disease. [32.]

The mechanism of how the SOD1 denaturation and aggregation leads to neuron and myelin degeneration in the spinal cord of dogs is not fully understood. There is evidence of higher O_2^- concentrations in the cells expressing the E40K-SOD1, which could suggest elevated oxidative stress due to interference in the enzymatic activity of the protein. However, recombinant E40K-SOD1 has been witnessed to have similar enzymatic activity to the wild type. Enzymatic activity has not yet been evaluated endogenously. Other types of mechanisms might explain increased O_2^- levels as well, such as respiratory complex I inhibition at the inner mitochondrial membrane. Regardless of the mechanism, O_2^- is highly reactive and cytotoxic since it damages DNA and the phospholipid membrane of cells which could explain spinal neuron degeneration in DM. Alterations in mitochondrial morphology and lower ATP concentrations have been witnessed as well in cells expressing E40K-SOD1, suggesting interference in mitochondrial function. [33.]

Another missense mutation, *SOD1*:c.52A>T, in exon 1 of the *SOD1* has been associated with the risk of DM but only in Bernese Mountain Dog [34]. This allele has not been found in any other breed or mixed-breed dogs [35]. In addition, a modifier locus in the *SP110* gene located in chromosome 25 that encodes SP110 nuclear body protein has been identified in Pembroke Welsh Corgi. SP110 participates in the regulation of gene transcription and certain haplotype within the *SP110* has been associated with increased risk and earlier onset of DM. [36.] Similar yet unidentified modifier genes in other breeds could explain the differences between different breeds on A/A homozygosity and risk of DM.

2.2.3 Epidemiology & Population Genetics

Data on the prevalence of DM in dog populations based on clinical diagnosis are limited. This could be because the symptoms of the disease resemble other similar diseases and the diagnosing is laborious, always demanding a histopathological sample from the spinal cord [37]. According to North American Veterinary Colleges data, the reported prevalence of DM in the overall dog population completely based on clinical signs was 0.19 % and the most DM-affected breeds were German Shepherd Dog, Welsh Corgi, Cardigan, Chesapeake Bay Retriever, Rhodesian Ridgeback, Irish Setter, Boxer, Pembroke Welsh Corgi, Wire Fox Terrier, Collie, and Old English Sheepdog in 1999 (Table 1). In addition, mixed-breed dogs have been reported to be affected. [38.]

Table 1. DM prevalence in all dogs and different breeds is based solely on clinical signs [38].

Breed	**DM-Affected Dogs (%)**
All dogs	0.19
German Shepherd Dog	2.01
Cardigan Welsh Corgi	1.51
Chesapeake Bay Retriever	0.83
Rhodesian Ridgeback	0.74
Irish Setter	0.68
Boxer	0.59
Pembroke Welsh Corgi	0.58
Wire Fox Terrier	0.52
Collie	0.38
Old English Sheepdog	0.38
Mixed breed	0.15

Data regarding the prevalence of the DM-associated *SOD1*:c.118G>A allele A is much newer and more comprehensive. The largest canine genotyping study up to date (N = 1 054 293), including 267 breeds and mixed breed dogs mostly from the USA (93.6 % of the samples) found, that DM-associated *SOD1* allele was the 2nd most prevalent disease allele in the overall dog population in August 2021. Allele A frequencies were found to be 7.9 %; 7.5 %; and 9.0 % in all, mixed breed, and purebred dogs, respectively. The disease allele was found most frequently in Wire Fox Terrier, Norfolk Terrier, Broholmer, English Toy Spaniel, Pembroke Welsh Corgi, Cavalier King Charles Spaniel, Bergamasco, Medium Poodle, Portuguese Pointer, and Boxer (Table 2). Genotype frequencies for G/G homozygosity, A/G heterozygosity, and A/A homozygosity were found to be 85.5 %; 13.3 %; and 1.2 % in all, 85.8 %; 13.3 %; and 0.9 % in mixed breed, and 84.4 %; 13.2 %; and 2.4 % in purebred dogs, respectively. [11.]

Table 2. *SOD1*:c.118G>A allele A frequency in different breeds [11].

Breed	**Allele A Frequency (%)**
Wire Fox Terrier	82.4
Norfolk Terrier	76.5
Broholmer	75.0
English Toy Spaniel	75.0
Pembroke Welsh Corgi	53.3
Cavalier King Charles Spaniel	51.0
Bergamasco	50.0
Medium Poodle	50.0
Portuguese Pointer	50.0
Boxer	42.1

2.3 KASP SNP Genotyping

Competitive allele-specific PCR (KASP) is a fluorescent genotyping method based on PCR that can be used to detect small genetic variations between homologous DNA sequences: SNPs, insertions, or deletions. It can be used to characterize bi-allelic loci that a diploid organism is carrying and hence genotype the subject of interest in relation to two alleles. [39; 40.]

The main reagents of KASP SNP genotyping reactions are the template DNA containing the SNP of interest, one common reverse primer, two allele-specific forward primers, and two fluorescence resonant energy transfer (FRET) cassettes, both containing unique 5'-fluorescein-labeled oligo bound to 3'-quencher-labeled oligo. Allele-specific forward primers have unique unlabeled tails at the 5’-end and complementary sequences for the SNP of interest at the 3’-end. Fluorescein oligos are labeled with either fluorescein amidite (FAM) or hexachlorofluorescein (HEX) and both are identical to one of the tails of the two forward primers. Both quencher oligos are complementary to one of the fluor-

labeled oligos. Fluorescein and quencher oligos are initially bound together to prevent any unwanted signal generation of the fluorescein dyes before attachment to the template. [39; 40.]

Two allele-specific forward primers and the common reverse primer are in the SNP-specific KASP assay mix. The FRET cassettes, Taq polymerase, free nucleotides, $MgCl_2$ cofactor, and a passive reference dye, carboxy-X-rhodamine (ROX), are in the universal KASP master mix. [40.] ROX is used to emit a stable fluorescence signal that is independent of the template concentration throughout the PCR cycles to normalize the fluorescence signal of the samples and hence reduce the variability caused by errors such as pipetting mistakes or PCR instrument issues. [41.]

LGC Genomics is the main supplier of KASP reagents. When ordering unvalidated custom KASP assay mixes for SNP genotyping, genomic data for the SNP of interest needs to be provided for LGC Genomics. Corresponding primer sequences are then designed, synthesized, and provided to the customer. [39; 40.]

The onset of the KASP reaction can be divided into 3 important initial PCR cycles. In the 1st cycle, the template-DNA is denatured and an allele-specific primer, that has a complementary nucleotide at the 3'-end for the SNP of interest found in the template, binds to the sequence flanking the SNP upstream, the last nucleotide directly binding to the SNP at the 5'-end of the template. The common reverse primer binds to the other template strand. [39; 40.] By binding to the template the primers create short, double-stranded sequences with a free —OH group at the 3'-end to which DNA polymerase can attach new nucleotides, hence creating a starting point for the synthesis of the complementary copies of the template [42]. Elongation takes place, incorporating the allele-specific primer into the template, the tail still staying unbound. In the 2nd cycle, the amplicon with the complimentary copy of the allele-specific primer tail gets synthesized for the first time with the help of the reverse primer, incorporating the unique allele-specific primer tail into the

template as well. In the 3rd cycle, a fluorescein oligo corresponding to the allele-specific primer tail is cleaved from the quencher oligo and bound to the complementary tail sequence in the template, and a fluorescence signal is generated for the first time. In the following cycles, the fluorescein oligos binding to the complementary tail sequence are increased exponentially along with their emitted fluorescence signal. If the genotype regarding the specific SNP is homozygous, only one fluorescein signal (FAM or HEX) is detected. If the genotype is heterozygous, signals of FAM and HEX are both detected. [39; 40.] Fluorescein signals of each sample are plotted as individual data points in a scatter plot in which the X-axis represents the FAM and the Y-axis the HEX signal [43]. The principles of KASP are explained in Figure 2.

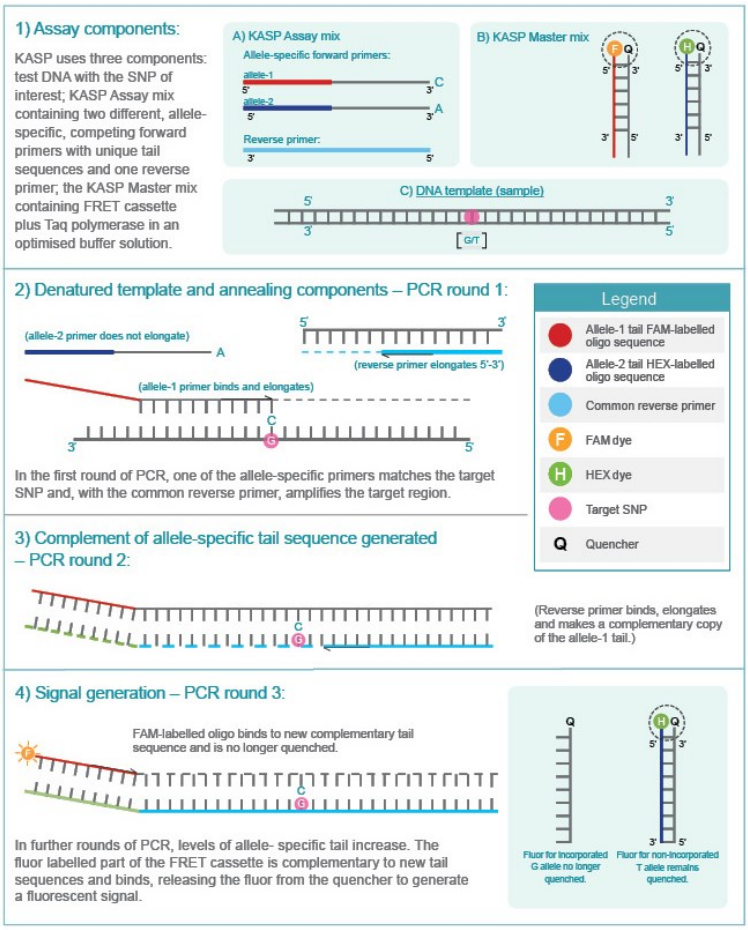

Figure 2. The principles of KASP [40].

Since the DM-associated mutation, *SOD1*:c.118G>A is a SNP, KASP could be utilized in genotyping the dog of interest in relation to the two alleles, G and A,

of the *SOD1*. The use of KASP has been previously reported in this context [44; 45].

3 Materials and Methods

3.1 Sample Collection

According to the findings of Donner et al., the breed with the highest DM-associated *SOD1*:c.118G>A allele A frequency is the Wire Fox Terrier [11]. Hence, most of the dogs selected for this study were of this breed to increase the chance of detecting this genetic variation. Two buccal swab samples were collected from each dog. Samples were collected from 10 Wire Fox Terriers, 2 Finnish Lapphunds, and 1 Stabyhoun and marked with sample ID (Dog 1−13). For the assessment of the reproducibility of the genetic test, 4 additional buccal swab samples were collected from one Finnish Lapphund (Dog 2). Wire Fox Terrier samples were sourced via the Finnish Fox Terriers Association's Breeding Commission and the rest via acquaintances of the author. Samples were asked to be taken from unrelated Wire Fox Terriers to ensure high genetic variation. All samples were collected from dogs that are not known to suffer from DM.

Sample collection protocol, two buccal swabs in a sterile package (Puritan Medical Products Company LLC, Guilford, USA), a swab storage bag, a customer information form, and an envelope to be returned to the laboratory were sent to the owners via mail.

Illustrated sample collection protocol was created in Finnish (Appendix 1). The protocol was based on the buccal swab sample collection protocols of several canine genetic testing companies and Medigoo’s sample collection protocol for human buccal swab samples [46–51]. In the protocol, owners were instructed to follow several measures before sampling to prevent sample contamination with foreign dog DNA or other contaminants. Owners were instructed to prevent the dog from eating, chewing shared or toys of other dogs, or having close contact

with another dog 60 minutes before sampling. In addition, owners were instructed to prevent puppies from being breastfed 60 minutes before sampling. In addition, it was instructed to take samples from one dog at a time and wash hands between the dogs if samples were taken from several dogs. To collect the buccal swab samples, owners were instructed to gently scrub and rotate the swab against the upper cheek for 15 seconds while avoiding teeth to prevent contamination, and to repeat this on the other side with another clean swab. After the sampling, the buccal swabs were instructed to be dried in room temperature for 1.5 hours to prevent mold growth and then placed in a swab storage bag, which had to be then placed in an envelope along with a customer information form to be returned to the laboratory via mail.

3.2 DNA Isolation

Two DNA isolation protocols, Protocol 1 (P1) and Protocol 2 (P2), were tested for each sampled dog to assess possible differences between DNA yield, purity, and performance in KASP. P1 had been tested, validated, and used at Medigoo for human buccal swab samples before. Both protocols were based on DNA isolation kit's (Genekam Biotechnology AG, Duisburg, Germany) protocol for buccal swab samples, with slight alterations. In Genekam's original protocol, the swab top is inserted into the sample tube before 7 minutes of incubation. In P1, this step had been removed, and the swab was thoroughly rotated and swished in the tube instead and discarded before incubation, and the incubation time was reduced to 5 minutes to make the protocol faster and less laborious. Furthermore, in Genekam's original protocol in the end possible contaminants are precipitated, and the supernatant containing the DNA is separated into a clean tube. This step was removed from P1 and P2 due to the lack of proper centrifuge at the laboratory. Otherwise, P2 was kept identical to the original protocol. The Genekam DNA isolation kit consists of 3 reagents: NaOH solution (Solution A), buffer (Solution B), and solvent (Solution C).

In the protocol P1 190 µl of Solution C and 10 µl of Solution A were pipetted into 2 ml tubes marked with sample IDs. Samples were vortexed for 5 seconds.

Swabs were inserted into the tubes, rotated, swished around for 15 seconds, and discarded. Tubes were vortexed for 5 seconds and incubated at 88 °C for 5 minutes. Tubes were vortexed for 4 x 5 seconds during the incubation. After the incubation, the tubes were centrifuged with a mini centrifuge for 2 seconds. 150 µl of Solution B was pipetted into each tube. Tubes were vortexed for 5 seconds. Each stock solution was diluted to a working solution in nuclease-free H_2O in a ratio of 1:20. Stock and working solutions were stored at 4 °C upon use.

In the protocol P2 190 µl of Solution C and 10 µl of Solution A were pipetted into 2 ml tubes marked with sample IDs. Samples were vortexed for 5 seconds. Tops of the swabs were cut with scissors, decontaminating the scissors with RNase AWAY decontaminant (Molecular BioProducts Inc., San Diego, USA) before usage and between each sample. Swab tops were inserted into the tubes. Tubes were vortexed for 5 seconds and incubated at 88 °C for 7 minutes. Tubes were vortexed for 4 x 5 seconds during the incubation. After the incubation, the tubes were centrifuged with a mini centrifuge for 2 seconds. 150 µl of Solution B was pipetted into each tube. Tubes were vortexed for 5 seconds. Swab tops were removed from the tubes with tweezers, decontaminating the tweezers with RNase AWAY before usage and between each sample. Stock solutions were diluted to a working solution in nuclease-free H_2O in a ratio of 1:20. Stock and working solutions were stored at 4 °C upon use.

After the isolation, the DNA concentrations (ng/µl) and ratios of absorbances (A_{260}/A_{280} and A_{260}/A_{230}) to assess purity were determined for each sample (see Table 4 and Figure 3 later in the text) with a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A mixture of solutions A, B, and C with a ratio corresponding to the samples was used as a blanking solution. A blanking cycle was performed before measurements to ensure that the instrument was working correctly and that there was not any sample carryover from previous measurements. Each sample was measured in triplicates and the mean was determined. To assess measurement

reproducibility, standard deviations for samples with concentrations of 2–100 ng/µl and coefficients of variations (CV%) for samples with concentration >100 ng/µl, were determined [52].

Due to the small sample size ($n<30$) and uncertainty of the normal distribution of the data, a two-tailed Wilcoxon signed-rank test was selected to test possible statistically significant differences in DNA yield and purity (A_{260}/A_{280} and A_{260}/A_{230}) between the paired dog samples isolated with P1 and P2 (see Appendix 3) [53, p. 19–20, 34]. The null and alternative hypotheses were stated as H_0: The median of differences in DNA yield/purity between the paired dog samples is 0. H_1: The median of differences in DNA yield/purity between the paired dog samples is not equal to 0. [53; 54.]

3.3 KASP Assay

Reference SNP cluster ID (rsID), flanking sequence 100 bp upstream and downstream, and genomic location for *SOD1*:c.118G>A (Table 3) were sourced from the Ensembl genome database and sent to LGC Genomics for custom KASP assay mix designing [28].

Table 3. Specifications of *SOD1*:c.118G>A SNP, including rsID, organism, alleles, genomic location, and flanking sequence in 5'-3' direction, that were sent to LGC Genomics [28].

rsID	rs853026434
Organism	*Canis lupus familiaris* (Dog)
Allele 1	G
Allele 2	A
Genomic location (CanFam 3.1)	Chromosome 31: g.26540342G>A
Sequence (5'-3')	TTCTTTGTTC AGAAGCACTT GCTCTCTCAT TTTTTGTGCT TTTCTTTGAC TGAAGGGAAG TGGGCCTGTT GTGGTATCAG GAACCATTAC AGGGCTGACT [G/A] AAGGCGAGCA TGGATTCCAC GTCCATCAGT TTGGAGATAA TACACAAGGT GGGTGTTGTG TTGGTCTAGT GACTCTTCTA TTTGTTTCAT CTAGTAAGAT

The primers of KASP assay mix were designed by LGC Genomics and sent to Medigoo via mail. Allele A was specific to FAM and Allele G for HEX. After the arrival, the KASP assay mix (LGC Genomics, London, UK) was stored at -20 °C before use.

3.4 KASP Protocol

For each KASP reaction, 0.084 µl of KASP assay mix (LGC Genomics, London, UK) and 3 µl of KASP master mix (LGC Genomics, London, UK) were mixed, and 3 µl of this mixture was pipetted into each marked well of an optical 96-well

plate. 3.5 µl of sample working solution was pipetted into each well. Each sample was included in duplicates in the plate to assess assay repeatability. Two no template controls (NTC) were included in the plate. NTCs contained the KASP assay mix and KASP master mix mixture without the template DNA. The plate was sealed with a special optical adhesive seal, centrifuged with microplate microcentrifuge for 5 seconds, and loaded into AriaMx Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA). The KASP 61-55 °C thermal cycling protocol with 40 amplification cycles was used and followed by the KASP recycling protocol [55]. FAM and HEX fluorescence signals (ΔRn) were determined for each sample and graphically displayed in a scatter plot with the instrument's software (see Figure 4 later in the text). The equivalency of duplicates and lack of signal of NTCs was checked. Samples were genotyped as G/G, G/A, or A/A based on their fluorescence signals. Another plate with 4 replicates of Dog 2 and NTC was made to assess the reproducibility of the genetic test. 2 replicates were isolated with P1 and 2 replicates with P2.

4 Results

4.1 DNA Concentrations and Purities

Triplicate measurements of samples with a concentration of 2–100 ng/µl had a standard deviation within ± 2 ng/µl and samples with a concentration of >100–15 000 ng/µl had ≤ 2 % coefficient of variation (CV%) and therefore had expected measurement reproducibility determined by the instrument manufacturer [52]. The only exception was Dog 1, isolation P2 whose measurements had higher CV% and were therefore discarded. In addition, the concentration for Dog 13, P1 isolation was below the limit of detection (LOD) of the instrument (<2 ng/µl) and was therefore discarded. DNA concentrations (ng/µl) and purities (A_{260}/A_{280} and A_{260}/A_{230}) could be determined for both isolations (P1 and P2) of Dogs 2–12 (Table 4 and Figure 3). See Appendix 2 for the full measurement data.

Table 4. Summarized data from the measured DNA concentrations (ng/µl) and purities (A_{260}/A_{280} and A_{260}/A_{230}) of Dogs 2–12 isolated from the duplicate buccal swabs with DNA isolation protocols P1 and P2. Means ($\bar{X}$) express the average DNA yields and purities for both protocols and mean differences ($\bar{X}_d = \bar{X}_{P2} - \bar{X}_{P1}$) how much DNA yields and purities of protocols differ from each other on average. Standard deviations (σ), coefficients of variations (CV%), and ranges (Min value–Max value) summarize how much the yields and purities vary with both isolation protocols. The full measurement data can be found in Appendix 2.

	Concentration (ng/µl)		**Purity (A_{260}/A_{280})**		**Purity (A_{260}/A_{230})**	
	P1	**P2**	**P1**	**P2**	**P1**	**P2**
$\bar{X}$	51.85	249.75	1.66	1.60	0.46	0.35
$\bar{X}_d$	197.91		-0.065		-0.11	
Range	11.33–176.07	56.17–603.87	1.46–1.99	1.39–2.06	0.38–0.61	0.24–0.47
σ	45.85	151.40	0.15	0.20	0.06	0.06
CV%	88.4 %	60.6 %	8.9 %	12.8 %	13.5 %	18.4 %

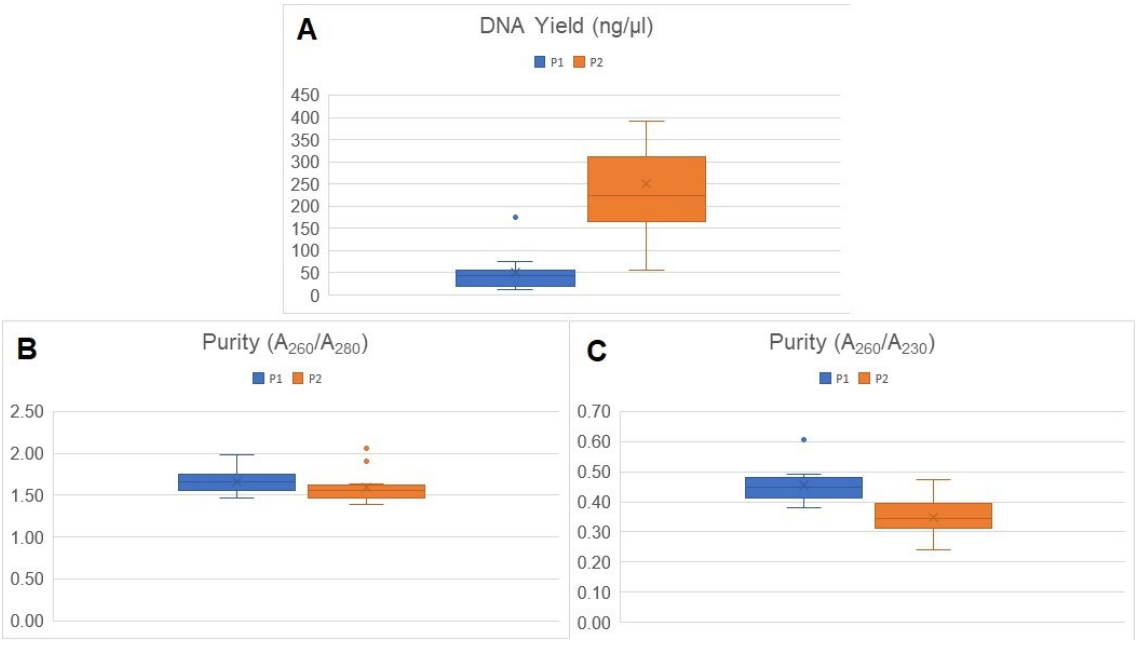

Figure 3. Boxplots of A: DNA concentrations (ng/µl), B: DNA purities (A_{260}/A_{280}), and C: DNA purities (A_{260}/A_{230}) of samples of Dogs 2–12 isolated from the duplicate buccal swabs with protocols P1 and P2. (Dog 4, P2 isolation with a concentration of 603.87 ng/µl excluded from Diagram A as an outlier).

Concentrations of dog samples isolated with P2 were consistently higher and the mean difference between P2 and P1 data sets was notable (197.9 ng/µl). There was no noticeable difference in A_{260}/A_{280} (0.07). There was a slight difference in A_{260}/A_{280} (0.11) in favor of P1 and samples isolated with P1 had a consistently higher ratio. Differences in concentrations and A_{260}/A_{230} between the paired dog samples isolated with P1 and P2 were confirmed to be statistically significant with the two-tailed Wilcoxon signed-rank test (W < Critical Value) with a 99% confidence level (See Appendix 3).

The concentration of the 2nd technical replicate of Dog 2, P1 isolation was below the LOD of the instrument (<2 ng/µl) and was therefore discarded. Otherwise, the technical replicates of Dog 2 could be measured (Table 5).

Table 5. DNA concentrations, purities (A_{260}/A_{280} & A_{260}/A_{230}), and standard deviations (σ) of the technical replicates of Dog 2 that were isolated from the duplicate buccal swabs with protocols P1 and P2. The measured concentration for Replicate 2, P1 isolation was below the LOD of the instrument and therefore the concentration and corresponding purity values were discarded.

	Concentration (ng/µl)		**Purity (A_{260}/A_{280})**		**Purity (A_{260}/A_{230})**	
Replicate	**P1**	**P2**	**P1**	**P2**	**P1**	**P2**
1	11.33	78.87	1.99	2.06	0.61	0.24
2	N/A	80.40	N/A	1.82	N/A	0.23
3	7.83	88.60	1.65	1.66	0.30	0.26
σ	2.47	5.23	0.24	0.20	0.21	0.02

Concentrations of the technical replicates were close in value, and were consistently higher with P2, and standard deviations were of the same magnitude with P1 and P2. However, because one of the technical replicates isolated with P1 was below the instrument's LOD and could not be measured, there is some uncertainty in the results. A_{260}/A_{230} of the technical replicates had some experimental error with the P1 samples, but the P2 samples were close in

value. The experimental error could explain the slight difference in A_{260}/A_{230} between P1 and P2.

4.2 KASP SNP Genotyping Results

FAM and HEX fluorescence signals (ΔRn) and corresponding genotypes could be determined for all samples (Table 6 and Figure 4).

Table 6. Determined *SOD1*:c.118G>A genotypes for both isolations (P1 and P2) of Dogs 1–13 and their breeds.

Dog	**Breed**	**Genotype (P1)**	**Genotype (P2)**
1	Finnish Lapphund	GG	GG
2	Finnish Lapphund	GG	GG
3	Wire Fox Terrier	AA	AA
4	Wire Fox Terrier	AA	AA
5	Wire Fox Terrier	AA	AA
6	Wire Fox Terrier	AA	AA
7	Wire Fox Terrier	AA	AA
8	Wire Fox Terrier	AG	AG
9	Wire Fox Terrier	AA	AA
10	Wire Fox Terrier	AA	AA
11	Wire Fox Terrier	AA	AA
12	Wire Fox Terrier	AG	AG
13	Stabyhoun	GG	GG

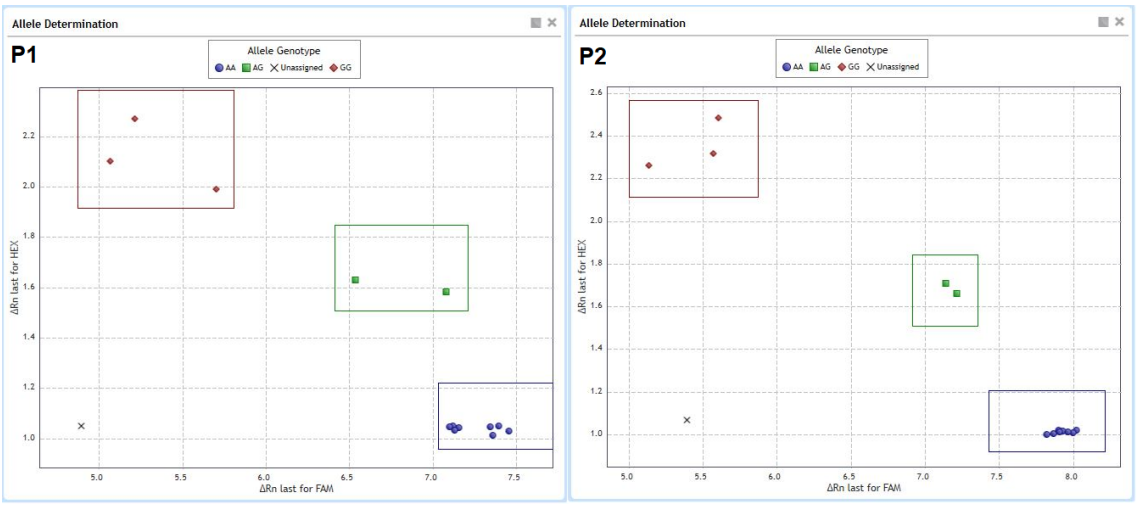

Figure 4. FAM (X-axis) and HEX (Y-axis) fluorescence signals (ΔRn) from the samples of Dogs 1–13 and their marked *SOD1*:c.118G>A allele genotypes based on their fluorescence signals. Red tilted squares represent the G/G, green squares the A/G, blue circles the AA genotype, and grey x the NTC. P1: Samples for which the DNA isolation protocol P1 was used. P2: Samples for which the DNA isolation protocol P2 was used.

Dog samples isolated with P1, genotyped as G/G, had approximately a HEX ΔRn within 2.0–2.3 and a FAM ΔRn within 5.0–5.75. A/Gs had approximately a HEX ΔRn of 1.6 and a FAM ΔRn within 6.5–7.0. A/As had approximately a HEX ΔRn of 1.1 and a FAM ΔRn within 7.0–7.5. NTC had approximately a HEX ΔRn of 1.1 and a FAM ΔRn of 5.0.

Dog samples isolated with P2, genotyped as G/G had approximately a HEX ΔRn within 2.2–2.5 and a FAM ΔRn within 5.0–5.5. A/Gs had approximately a HEX ΔRn within 1.6–1.7 and a FAM ΔRn within 7.0–7.25. A/As had approximately a HEX ΔRn of 1.00 and a FAM ΔRn within 7.75–8.0. NTC had approximately a HEX ΔRn of 1.1 and a FAM ΔRn of 5.5.

The only difference between samples isolated with P1 and P2 DNA isolation protocols was that the sample set P2 exhibited slightly higher signals and tighter clusters in the scatter plot. The repeatability of the results was 100% since duplicate samples and samples isolated with different protocols from the same dog yielded comparable signals and were determined to be of the same

genotype. While NTCs exhibited some signals, they were separated from the sample clusters. Sample genotypes were organized in Table 7.

Table 7. *SOD1*:c.118G>A genotype frequencies of the Dogs 1–13.

Breed	**Dogs Genotyped**	**G/G**	**A/G**	**A/A**
Wire Fox Terrier	10	0	2	8
Finnish Lapphund	2	2	0	0
Stabyhoun	1	1	0	0

Of the 10 genotyped Wire Fox Terriers 8 were A/A homozygous and 2 A/G heterozygous. 2 Finnish Lapphunds and 1 Stabyhoun were all G/G homozygous.

FAM and HEX fluorescence signals (ΔRn) and corresponding genotypes could be determined for all technical replicates of Dog 2 as well (Figure 5).

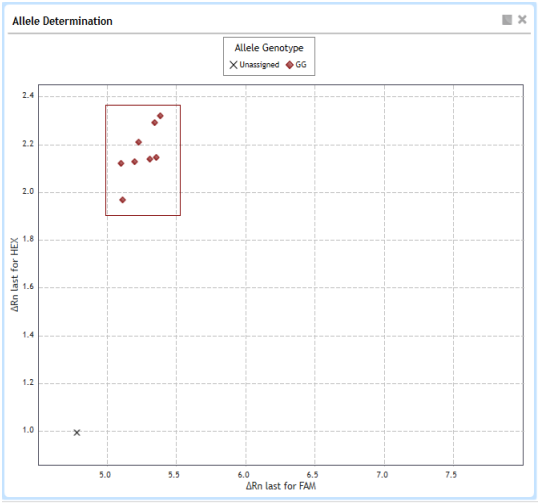

Figure 5. FAM (X-axis) and HEX (Y-axis) fluorescence signals (ΔRn) of the 4 additional technical replicates of both isolations (P1 and P2) of Dog 2 included in duplicates, and their marked *SOD1*:c.118G>A allele genotypes based on their fluorescence signals. Red tilted squares represent the G/G genotype, and the grey x is the NTC.

Technical replicates of Dog 2, genotyped as G/G, had approximately a HEX ΔRn within 2.0–2.4 and a FAM ΔRn within 5.0–5.5. NTC had approximately a HEX ΔRn of 1.00 and a FAM ΔRn of 4.75. The repeatability of the replicates was 100% since all technical replicates of Dog 2 produced comparable signals and were determined to be of the same genotype. While NTC exhibited some signal, it was separated from the sample cluster.

5 Conclusions and Discussion

The aim of this bachelor's thesis was to establish a framework for the development of animal genetic tests for Medigoo and develop their first commercially available clinical genetic test for an animal. A genetic test for the main causative mutation of canine DM was developed [8].

A literature review was done on the regulatory environment for animal-targeted genetic tests and their development to understand what kind of genetic tests are legal to develop and sell, and with what conditions. Development and selling of clinical genetic tests for animals are currently mildly regulated in the EU and Finland. Genetic tests such as this one can be freely developed and sold, if they are appropriate for their purpose, animal owners are well informed with clear sample collection protocols in a way that sample collection does not cause any pain or harm to the animal, and buccal swab DNA samples are used instead of blood samples. [20–22.]

In addition to regulatory information, the literature review provides Medigoo with sufficient information to understand DM and inform potential customers about it: its clinical signs and disease progression, genetics and pathogenesis, the prevalence of the disease in different breeds as well as disease allele and genotype frequencies. In summary, DM is a neurodegenerative disease characterized by SOD1 aggregation, oxidative stress as well as motor neuron and myelin loss in the spinal cord of dogs [10; 25; 31; 33]. It is a paralyzing and eventually fatal disease. It is an autosomal recessive disease with incomplete penetrance linked to major causative mutation, *SOD1*:c.118G>A [8; 23].

SOD1:c.52A>T in Bernese Mountain Dog and specific haplotype within *SP110* in Pembroke Welsh Corgi have been associated to DM as well [34; 36]. The genotyping study of Donner et al. is the newest and most comprehensive study up to date that provides statistics about DM-associated allele and genotype frequencies. It can be used as a reference when assessing how well the genotyping results of this specific KASP assay used in this test compare to the genotypic distribution (G/G: 85.5 %; A/G: 13.3 %; A/A: 1.2 %) and A allele frequency (7.9 %) of the *SOD1*:c.118G>A in all dogs or specific breeds. [11.]

Specifications for *SOD1*:c.118G>A, such as rsID, genomic location, and flanking sequence were sourced from the Ensembl database, CanFam 3.1 dog genome assembly for the ordering of the KASP assay [28]. Medigoo can use the database in the development of other similar genetic tests for genes linked with canine diseases in the future. Furthermore, the Online Mendelian Inheritance in Animals (OMIA) database used as a source in this thesis contains hundreds of Mendelian diseases with at least one known causal variant for pets and other domestic animals, which could be useful in the future for the development of animal genetic tests [4].

The KASP SNP genotyping assay mix for the detection of the alleles of *SOD1*:c.118G>A was ordered and tested with 13 duplicate dog buccal swab samples isolated with two different DNA isolation protocols. A sample collection protocol for canine buccal swab samples was made. Two DNA isolation protocols, P1 and P2, were tested for the samples. While protocol P2 consistently yielded more DNA than P1, and this difference was confirmed to be statistically significant, no notable difference was found in purity or performance in KASP. 2 out of 15 P1 isolations had concentrations lower than Nanodrop's LOD. This problem did not arise with samples isolated with P2. However, even these samples performed sufficiently in KASP and could be genotyped.

DNA absorbs at 260 nm, proteins at 280 nm, and contaminants such as carbohydrates and phenols at 230 nm. Pure DNA typically has A_{260}/A_{280} of 1.8 and A_{260}/A_{230} of 2.0-2.2. [56.] Most of the samples had slightly lower A_{260}/A_{280}

and notably lower $A_{260}/A_{230,}$ indicating the presence of proteins and other contaminants in the samples, probably due to the missing centrifugation step at the end of both protocols. However, this did not raise any issues in KASP, since all samples had sufficient, clearly interpretable, and repeatable fluorescence signals.

Based on findings on the differences between P1 and P2, there appears to be no grounds for the use of the more time-consuming, laborious, and expensive P2 isolation protocol, and the original P1 protocol that previously had been used for human buccal swab samples at Medigoo appears to work sufficiently and could be used for dog buccal swab samples as well. In addition, the impurities in the samples do not affect performance in KASP and the centrifugation step at the end of the original Genekam's protocol is not required. However, further testing with a larger number of dog samples and breeds is necessary to confirm the performance of P1 across the dog population.

Furthermore, since all collected samples had sufficient purity and amount of DNA for KASP, the sample collection protocol worked as intended and was sufficiently comprehensible for dog owners who participated in this study to follow correctly.

According to Donner et al., Wire Fox Terrier has the highest DM-associated *SOD1*:c.118G>A allele A frequency [11]. Most of the dogs selected for this study were of this breed to increase the chance of detecting the disease allele with the test. This decision provided expected results since 8 of the 10 sampled Wire Fox Terriers were genotyped to be A/A homozygous and 2 out of 10 A/G heterozygous with the test. The high allele A frequency of Wire Fox Terriers determined with this test is in line with the findings of Donner et al. and other genotyping studies (82.4–94.0 %) [11; 35]. In contrast, allele A frequency has been found to be relatively low in Finnish Lapphunds (14.9 %) and Stabyhouns (11.1 %), which all were genotyped to be G/G homozygous with the test [11]. While the genotypes determined by the test are in line with independent

genotyping studies, the small sample size limits the ability to draw strong conclusions about the accuracy of the test in this regard.

The ethicality of the test should be considered. When dog owners, such as potential customers of the test and participants of this thesis are in the future informed about the genotyping results of the test, they should be provided with transparent, comprehensible, and honest reports. It should be highlighted, that while the association of *SOD1*:c.118G>A A/A homozygosity to DM in certain breeds has strong evidence, the disease does not have full penetrance, meaning that not all A/A homozygotes develop the disease [8; 23]. Age and breed are both affecting factors in the development of the disease [8; 10; 38]. In addition, unknown environmental and other genetic factors may play a role in the disease expression. *SOD1*:c.118G>A allele A has been found most prevalently in Wire Fox Terriers, thus most of the dogs selected for this study were of this breed to increase the chance of detecting the disease variant with the test [11]. 8 out of the 10 sampled Wire Fox Terriers were determined to be A/A homozygous even though all sampled dogs were known to be healthy. While *SOD1*:c.118G>A allele A distribution in dog populations has been studied widely and has been found most prevalently in Wire Fox Terriers, currently there are no breed-specific studies on the association of *SOD1*:c.118G>A allele A to DM in Wire Fox Terriers, even strong association has been confirmed in Boxer, Pembroke Welsh Corgi, German Shepherd Dog, Chesapeake Bay Retriever, and Rhodesian Ridgeback [8; 11]. This should be highlighted especially for the Wire Fox Terrier owners. In addition, it should be considered if the test should be sold only for the breeds for which there is evidence of this association in the literature. Furthermore, the test has not been developed for veterinary use and should not be used for diagnostic purposes. While the test has potential as a tool in dog breeding, and there is some evidence that the introduction of such tests has decreased the prevalence of DM in dogs without affecting the genetic diversity, breeding decisions should not be based on such tests alone and no breeding advice should be provided along with the results [7; 13]. Dog owners could be provided with links to relevant research about DM to help them understand better the disease and the test itself.

The small sample size and low number of breeds (n = 13, 10 Wire Fox Terriers, 2 Finnish Lapphunds, and 1 Stabyhoun) used in this study limit the ability to conclude the test's performance in the general dog population. The test, including sample collection and DNA isolation protocols, and the KASP assay, works as intended with confidence with buccal swab samples of Wire Fox Terriers.

The accuracy, i.e., the closeness of the measured values to the true values, of the KASP assay for the detection of the alleles of *SOD1*:c.118G>A was not determined in this study and is yet to be verified [57]. However, KASP is generally considered a highly accurate genotyping method (99.8 % accuracy announced by LGC Genomics) [58]. Independent researchers have reported it to have a high accuracy (97.6–99.5 %) in different applications as well [59–62]. The accuracy of this specific assay could be verified by using different genotyping methods, such as sequencing, TaqMan assay, or PCR restriction fragment length polymorphism (PCR-RFLP) to verify if they produce comparable genotypes for the same samples [60; 62; 63]. In addition, positive controls could be used. However, sourcing them might be challenging.

The precision of an analytical method consists of repeatability and reproducibility [57]. The repeatability of an assay is defined as the ability to produce similar results for identical samples within the same laboratory using the same reagents, instruments, and protocols [64]. The KASP assay used in this study provided repeatable results since all samples included in duplicates on the 96-well plate, samples isolated from the same dog with different protocols, as well as technical replicates were determined to be of the same genotype. The precision of the test has been validated in this regard. In contrast, the reproducibility of an assay describes the assay's ability to produce similar results for identical samples, when the assay is performed by different laboratories with different analysts and instruments [57; 64]. This study did not validate completely the precision since the reproducibility of the test between different laboratories was not verified.

Now Medigoo has a framework that makes the development of genetic tests for dogs and possibly other animals faster and less laborious hereafter. In addition, Medigoo has now repeatable genetic test for canine DM that could be introduced to the constantly growing dog DNA testing market in the future. Due to the small sample size and low number of breeds used in this study, the performance of the genetic test could be assessed in the future with a bigger and more randomized sample, including a larger number of dogs and breeds. The genetic test could be expanded in the future by including assays for DM-associated *SOD1*:c.52A>T and *SP110* haplotype if the tested breed is Bernese Mountain Dog or Pembroke Welsh Corgi [34; 36]. Dog owners, who participated in this thesis shall be informed soon about the genotyping results described in Chapter 4.2 by creating and sending them a report that follows the ethical guidelines discussed earlier in this chapter.

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Appendix 1: Sample Collection Protocol

Ohjeet DNA-näytteen ottamiseen koirasta

Pakkaus sisältää:

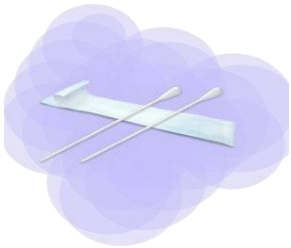
1. Näytteenottovälinepakkaus, jonka sisällä on 2 steriiliä näytteenottopuikkoa
2. Säilytyspussi näytteenottopuikoille
3. Näytteenotto-ohjeet
4. Asiakastietolomake
5. Valmiiksi maksettu palautuskuori näytteen laboratorioon lähettämistä varten

Ohjeet:

Oikean tuloksen varmistamiseksi näytteen tulee olla puhdas ja sen ei tule sisältää muiden kuin tutkittavan koiran DNA:ta. Täten koiran ei tule syödä, pureksia yhteisiä tai muiden koirien leluja tai olla lähikontaktissa toisen koiran kanssa 60 min ennen näytteenottoa. Lisäksi pentuja ei tule imettää 60 min ennen näytteenottoa. Jos näytteitä otetaan useammasta koirasta, ota näytteet yhdestä koirasta kerrallaan ja pese kätesi koirien näytteenottojen välissä.

1. Avaa näytteenottovälinepakkaus puikkojen kapeiden kärkien päädystä vetämällä auki pakkauksen sivuja siten, että pääset käsiksi puikkoihin. Avaa pakkaus noin puoleenväliin.

2. Poista ensimmäinen näytteenottopuikko pakkauksesta. Pyri pitämään koiran pää aloillaan ja nosta ylähuulta sen verran, että saat asetettua puikon ikenen ja posken väliin. Hankaa puikkoa posken sisäpintaa vasten 15 s ajan samalla pyörittäen. Älä käytä liiallista voimaa ja vältä hampaiden koskettamista. Aseta ensimmäinen puikko kuivumaan pakkauksen päälle poikittain siten, että puikon kärki säilyy ilmassa koskemattomana. Poista toinen puikko pakkauksesta ja toista näytteenotto vastakkaiselta puolelta suuta. Aseta toinenkin puikko kuivumaan. Kuivaa puikkoja 1,5 h ajan huoneilmassa.



3. Aseta näytteenottopuikot kuivauksen jälkeen säilytyspussiin pumpulikärki sisäänpäin. Säilytä pussi huoneenlämmössä suojassa suoralta auringonvalolta, kosteudelta ja painolta. Säilytyspussia ei saa laittaa muovipussiin.

4. Täytä ja allekirjoita asiakastietolomake. Jos palautat usean eläimen näytteet, täytä kaikkiin lomakkeisiin kohdat 4.–8. ja vain yhteen lomakkeista kohdat 1.–3.

5. Aseta näytteiden säilytyspussi ja asiakastietolomake palautuskuoreen ja sulje se. Postimaksu on valmiiksi maksettu, joten voit jättää sen lähimpään postilaatikkoon.

Appendix 2: DNA Concentrations and Purities – Full Data

DNA concentrations (ng/µl) and purities (A_{260}/A_{280} and A_{260}/A_{230}) could be determined for both isolations (P1 and P2) of Dogs 2–12 (Table 2.1).

Table 2.1. Measured DNA concentrations (ng/µl) and purities (A_{260}/A_{280} & A_{260}/A_{230}) of Dogs 2–12 isolated from the duplicate buccal swabs with DNA isolation protocols P1 and P2. Means ($\bar{X}$) express the average DNA yields and purities for both protocols and mean differences ($\bar{X}d = \bar{X}P2 - \bar{X}P1$) how much DNA yields and purities of protocols differ from each other on average. Standard deviations (σ) and coefficients of variations (CV%) summarize how much the yields and purities vary with both isolation protocols.

	Concentration (ng/µl)		**Purity (A_{260}/A_{280})**		**Purity (A_{260}/A_{230})**	
Dog	**P1**	**P2**	**P1**	**P2**	**P1**	**P2**
2	11.33	78.87	1.99	2.06	0.61	0.24
3	18.73	199.57	1.68	1.63	0.38	0.29
4	76.17	603.87	1.57	1.47	0.48	0.42
5	40.63	311.70	1.50	1.49	0.43	0.41
6	43.97	264.70	1.46	1.48	0.43	0.35
7	15.03	165.90	1.75	1.56	0.40	0.32
8	25.50	56.17	1.74	1.90	0.46	0.31
9	176.07	390.87	1.55	1.47	0.44	0.47
10	54.07	195.63	1.67	1.58	0.41	0.35
11	56.73	224.23	1.75	1.55	0.48	0.34
12	52.07	255.77	1.64	1.39	0.49	0.34
$\bar{X}$	51.85	249.75	1.66	1.60	0.46	0.35
$\bar{X}_d$	197.91		-0.065		-0.11	
σ	45.85	151.40	0.15	0.20	0.06	0.06
CV%	88.4 %	60.6 %	8.9 %	12.8 %	13.5 %	18.4 %

Concentrations of dog samples isolated with P2 were consistently higher and the mean difference between P2 and P1 data sets was notable (197.9 ng/µl).

There was no noticeable difference in A260/A280 (0.07). There was a slight difference in A260/A280 (0.11) in favor of P1 and samples isolated with P1 had a consistently higher ratio. Differences in concentrations and A260/A230 between the paired dog samples isolated with P1 and P2 were confirmed to be statistically significant with a two-tailed Wilcoxon signed-rank test ($W < \text{Critical Value}$) with a 99% confidence level (See Appendix 3).

Appendix 3: Wilcoxon Signed-Rank Tests

Two-tailed Wilcoxon signed-rank tests, a non-parametric version of the t-test, were executed as described by Riina. et al [65]. The hypotheses were stated as H0: The median of differences between the paired dog samples is zero and H1: The median of differences between the paired dog samples is not equal to zero [53; 54]. The differences (d = P1-P2) in DNA yield and purity between the two paired dog samples isolated with two different protocols, P1 and P2, were calculated. Negative signs (-1) were assigned for negative differences ($d<0$) and positive signs (1) for positive differences ($d>0$). This was followed by calculating the absolute values of the differences ($|d|$). Numerical ranks were assigned for each $|d|$ in ascending order: rank 1 being assigned to the lowest $|d|$. Signed ranks were determined by multiplying ranks with their corresponding sign. Next, negative and positive net sums ($T-$ & $T+$) were calculated as the sums of the ranks determined for the positive and negative differences, respectively. The final W-statistic was determined as the smaller of the $T-$ and $T+$. [54; 65.] Critical values were sourced from University of Florida's Critical Values of the Wilcoxon Signed Ranks Test table [66].

Wilcoxon signed-rank test for the difference in DNA yield

The hypotheses were stated as:

H0: The median of differences in DNA yield (ng/µl) between the paired dog samples is zero.

H1: The median of differences in DNA yield (ng/µl) between the paired dog samples is not equal to zero.

Wilcoxon signed-rank test for the difference in DNA concentrations (ng/µl) between the paired dog samples isolated with protocols P1 and P2 can be found in Table 3.1.

Table 3.1. DNA concentrations (ng/µl) of Dogs 2–12 isolated from the duplicate buccal swabs with DNA isolation protocols P1 and P2, corresponding signs of their differences, absolute values of differences (|d|), and signed ranks for the calculation of the test statistic (W).

Dog	P1 (ng/µl)	P2 (ng/µl)	Sign	|d|	Signed Rank
2	11.33	78.87	-1	67.53	-2
3	18.73	199.57	-1	180.83	-6
4	76.17	603.87	-1	527.70	-11
5	40.63	311.70	-1	271.07	-10
6	43.97	264.70	-1	220.73	-9
7	15.03	165.90	-1	150.87	-4
8	25.50	56.17	-1	30.67	-1
9	176.07	390.87	-1	214.80	-8
10	54.07	195.63	-1	141.57	-3
11	56.73	224.23	-1	167.50	-5
12	52.07	255.77	-1	203.70	-7

The calculated test statistic (W) for the Wilcoxon signed-rank test for the difference in DNA concentration (ng/µl) can be found in Table 3.2.

Table 3.2. Negative and positive net sums (T− & T+) calculated from the signed ranks of Table 3.1, test statistic (W) determined as the lower value of T+ and T-, number of pairs (n) and corresponding critical values of alphas (α) 0.05 and 0.01 for the n [66].

T+	0
T-	-66
W	0
n	11
Critical Value (α = 0.05)	10
Critical Value (α = 0.01)	5

W (0) < Critical Value (α = 0.01; 5) → H0 is rejected.

Conclusion: The paired dog samples isolated with different protocols, P1 and P2, differ by their DNA yield statistically significantly with a confidence level of 99%.

Wilcoxon signed-rank test for the difference in purity (A_{260}/A_{280})

The hypotheses were stated as:

H0: The median of differences in DNA purity (A_{260}/A_{280}) between the paired dog samples isolated with protocols P1 and P2 is zero.

H1: The median of differences in DNA purity (A_{260}/A_{280}) between the paired dog samples isolated with protocols P1 and P2 is not equal to zero.

Wilcoxon signed-rank test for the difference in DNA purity (A_{260}/A_{280}) between the paired dog samples isolated with protocols P1 and P2 can be found in Table 3.3.

Table 3.3. DNA purities (A_{260}/A_{280}) of Dogs 2–12 isolated from the duplicate buccal swabs with DNA isolation protocols P1 and P2, corresponding signs of their differences, absolute values of differences (|d|), and signed ranks for the calculation of the test statistic (W).

Dog	**P1 (A_{260}/A_{280})**	**P2 (A_{260}/A_{280})**	**Sign**	**|d|**	**Signed Rank**
2	1.99	2.06	-1	0.08	-4
3	1.68	1.63	1	0.05	3
4	1.57	1.47	1	0.10	7
5	1.50	1.49	1	0.01	1
6	1.46	1.48	-1	0.02	-2
7	1.75	1.56	1	0.19	9
8	1.74	1.90	-1	0.16	-8
9	1.55	1.47	1	0.08	5
10	1.67	1.58	1	0.09	6
11	1.75	1.55	1	0.20	10
12	1.64	1.39	1	0.25	11

The calculated test statistic (W) for the Wilcoxon signed-rank test for the difference in DNA purity (A_{260}/A_{280}) can be found in Table 3.4.

Table 3.4. Negative and positive net sums (T− & T+) calculated from the signed ranks of Table 3.3, test statistic (W) determined as the lower value of T+ and T-, number of pairs (n) and corresponding critical values of alphas (α) 0.05 and 0.01 for the n [66].

T+	52
T-	-14
W	14
n	11
Critical Value (α = 0.05)	10
Critical Value (α = 0.01)	5

W (14) > Critical Value (α = 0.05; 10) → H0 is not rejected.

Conclusion: The paired dog samples isolated with different protocols, P1 and P2, do not differ by their DNA purity (A_{260}/A_{280}) statistically significantly.

Wilcoxon signed-rank test for the difference in purity (A_{260}/A_{230})

The hypotheses were stated as:

H0: The median of differences in DNA purity (A_{260}/A_{230}) between the paired dog samples isolated with protocols P1 and P2 is zero.

H1: The median of differences in DNA purity (A_{260}/A_{230}) between the paired dog samples isolated with protocols P1 and P2 is not equal to zero.

Wilcoxon signed-rank test for the difference in DNA purity (A_{260}/A_{230}) between the paired dog samples isolated with protocols P1 and P2 can be found in Table 3.5.

Table 3.5. DNA purities (A_{260}/A_{230}) of Dogs 2–12 isolated from the duplicate buccal swabs with DNA isolation protocols P1 and P2, corresponding signs of their differences, absolute values of differences (|d|), and signed ranks for the calculation of the test statistic (W).

Dog	**P1 (A_{260}/A_{230})**	**P2 (A_{260}/A_{230})**	**Sign**	**|d|**	**Signed Rank**
2	0.61	0.24	1	0.37	11
3	0.38	0.29	1	0.09	7
4	0.48	0.42	1	0.06	3
5	0.43	0.41	1	0.02	1
6	0.43	0.35	1	0.07	5
7	0.40	0.32	1	0.08	6
8	0.46	0.31	1	0.15	9
9	0.44	0.47	-1	0.03	-2
10	0.41	0.35	1	0.06	4
11	0.48	0.34	1	0.14	8
12	0.49	0.34	1	0.15	10

The calculated test statistic for the Wilcoxon signed-rank test for the difference in DNA purity (A_{260}/A_{230}) can be found in Table 3.6.

Table 3.6. Negative and positive net sums (T− & T+) calculated from the signed ranks of Table 3.5, test statistic (W) determined as the lower value of T+ and T-, number of pairs (n) and corresponding critical values of alphas (α) 0.05 and 0.01 for the n [66].

T+	64
T-	-2
W	2
n	11
Critical Value (α = 0.05)	10
Critical Value (α = 0.01)	5

W (2) < Critical Value (α = 0.01; 5) → H0 is rejected.

Conclusion: The paired dog samples isolated with different protocols, P1 and P2, differ by their DNA purity (A_{260}/A_{230}) statistically significantly with a confidence level of 99%.