Using CRISPR/Cas9 to develop a serglycin deficient canine mastocytoma cell line

Preparation of future exploration of the role of serglycin in carcinogenesis

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Independent Project • 30 credits
Swedish University of Agricultural Sciences, SLU
Faculty of Veterinary Medicine and Animal Science
Veterinary Medicine Programme

Uppsala 2024

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Credits:	30 credits
Level:	Second cycle, A2E
Course title:	Independent Project in Veterinary Medicine
Course code:	EX1003
Programme/education:	Veterinary Medicine Programme
Course coordinating dept.:	Department of Clinical Sciences
Place of publication:	Uppsala
Year of publication:	2024

| **Keywords:** | cancer, canine, CRISPR/Cas9, dog, in vitro models, mastocytoma, PCR, serglycin |

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Abstract

Serglycin is a proteoglycan expressed by many different cells, especially mast cells, but also by cancer cells. It has been reported that the level of expression of serglycin in cancer cells corresponds to the malignancy of the tumor. The higher level of expression the higher malignancy and poorer prognosis. Serglycin can promote migration, metastasis and epithelial to mesenchymal transition by binding to an adhesion molecule on the cells. However, up to this point very few, if any, studies have addressed the functional roles of serglycin in canine mastocytoma.

In this study, a canine mastocytoma cell line was cultured. Mastocytoma (MCT) is one of the most common cutaneous neoplasia in dogs accounting for up to 21% of all canine skin tumors. Apart from the cutaneous form there is also a systemic form which typically affects the liver, spleen, and bone marrow, although this form is uncommon. The cutaneous MCTs have a wide range in their manifestation. They can be a slow growing lesion mistaken for a non-neoplastic change or fast growing, ulcerated lesion with inflamed surrounding tissue.

To allow future explorations of the role of serglycin in carcinogenesis, this study was aimed to develop serglycin deficient canine mastocytoma cells using CRISPR/Cas9. At the site of the double strand break (DSB) by Cas9 a gene cassette was inserted, using electroporation, containing genes for antibiotic resistance and fluorescence in green and red. The DSB and the correct incorporation of the gene cassette was verified in a fluorescence light microscope, with PCR reactions and selection with antibiotics. In the fluorescence microscope it was discovered that the cells were autofluorescent in both green and red. The PCR reactions confirmed the 5´ side of the gene cassette to be present in the transfected cells. However, no results were given when amplifying the whole gene cassette or the 3´ side. This could indicate that the gene cassette was fractured or that the method was not successful. The conclusion of this study was that further analysis, for example subcloning and qPCR, is necessary to determine if the knockout of the serglycin gene was successful in the cells.

Keywords: cancer, canine, CRISPR/Cas9, dog, in vitro models, mastocytoma, PCR, serglycin

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Abbreviations

C2	Cell line from mastocytoma in dog
CRISPR	Clustered regularly interspaced short palindromic repeats
Cas9	CRISPR associated (enzyme) 9
DSB	Double strand break
GFP	Green fluorescent protein
gRNA	Guide RNA
MCT	Mast cell tumor/ Mastocytoma
PCR	Polymerase chain reaction
RFP	Red fluorescent protein
SLU	Swedish University of Agricultural Sciences
SRGN	Serglycin
qPCR	Quantitative polymerase chain reaction

1. Introduction

Serglycin is a proteoglycan expressed by several cell types but is especially associated with storage and release of inflammatory mediators (Åbrink *et al*. 2004; Grujic *et al.* 2005; Braga *et al*. 2007; Niemann *et al*. 2007). Additionally, serglycin is expressed by cancer cells (Korpetinou *et al*. 2015). It has been shown that a high expression of serglycin is associated with more aggressive cancer types and therefore a poorer prognosis. Further, several types of cancers have been reported to be dependent on serglycin for its ability to metastasize (Li *et al*. 2011; Korpetinou *et al*. 2013; Roy *et al*. 2016; Guo *et al*. 2017). However, up to this point very few, if any, studies have addressed the functional roles of serglycin in canine mastocytoma.

Mastocytoma, or mast cell tumor (MCT), is a tumor originating from a white blood cell called mast cell. MCT is one of the most common cutaneous tumors in dogs and affects mostly older individuals (Hauck 2013; London & Thamm 2013; Willmann *et al*. 2019). Apart from the cutaneous form there is also a systemic form which typically affects the liver, spleen, and bone marrow (Welle *et al*. 2008). Systemic mastocytosis is a more aggressive cancer type and therefore have a poor prognosis. The cutaneous MCT have a wide range in their manifestation (London & Thamm 2013). They can be small lesions mistaken for a non-neoplastic change, a solitary slow growing lump, or fast growing, ulcerated with inflamed surrounding tissue.

In the present study, we will use CRISPR/Cas9 to develop serglycin deficient MCT cell lines from canine (C2). At the site of the double strand break (DSB) a gene cassette will be inserted, using electroporation, containing genes for antibiotic resistance and fluorescence in green and red. The future goal is to explore changes in morphology, growth, and metastasis, but also to identify which proteins and mediators that have been lost or changed in comparison to the wild-type C2. The purpose is to increase the understanding of serglycins role in the progress of cancer.

The methods used were cell culture, transfection with CRISPR/Cas9 and genomic polymerase chain reaction (PCR).

2. Literature review

2.1 Mastocytoma

A mastocytoma (MCT) is a tumor originating from a white blood cell called mast cell. The mast cell contains characteristic granules in the cytoplasm which stores several bioactive substances, including histamine, heparin, and tumor necrosis factor-alfa (TNF-alfa) (Henningsson *et al*. 2006). When stimulated, the mast cell can produce a number of proteases, cytokines, growth factors and other bioactive substances depending on its location in the body and is therefore involved in several processes (London & Seguin 2003; London & Thamm 2013). Examples of these processes are parasite reactions, hypersensitivity reactions, wound healing, induction of the congenital immune responses, and regulation of reaction to insect venoms (London & Thamm 2013). Mast cells are found in every organ throughout the body, with a large amount in the skin, conjunctiva, gastrointestinal and respiratory mucosa. Hence, MCT often occur at these locations, especially in the dermis (London & Seguin 2003; Fong & Crane 2023).

2.2 Mastocytoma in dogs

MCT is one of the most common cutaneous tumor in dogs and affects mostly older dogs, but without evident gender predilection (Hauck 2013; London & Thamm 2013; Willmann *et al*. 2019). A recent large study including 3139 canine skin tumors showed that MCT accounted for most cases of all skin tumors, *i.e.* approximately 14% (Śmiech *et al*. 2023). However, this number varies between countries and studies. MCT have been reported to account for up to 21% of all canine skin tumors (Welle *et al*. 2008; Kiupel 2016; Śmiech *et al*. 2023).

The etiology behind MCT is not clear, though underlying genetic causes are indicated since a few breeds are more likely to develop MCT, for instance Boxer, Shar Pei, bulldogs, Labrador and Golden retriever (London & Thamm 2013; Śmiech *et al*. 2019). In addition, several mutations in a gene called c-KIT have been determined in neoplastic mast cells (Willmann *et al*. 2019). The ligand of KIT, *i.e.*

stem cell factor, stimulates cell growth and maturation (Yanagihori *et al*. 2005). These mutations result in constitutive activation of the KIT receptor, causing ligand-independent proliferation of MCT cell lines in vitro. It has also been reportedin animal studies that these mutations can lead to tumorigenicity, which suggests that these mutations is a factor in the development of mastocytosis (Longley Jr. *et al*. 1999). A study including 56 cutaneous MCT found c-KIT mutations in 16% of the tumors (Webster *et al*. 2007). Though the incidence of c-KIT mutation varies from 13,6% to 45% in five different studies (London *et al*. 1999; Downing *et al*. 2002; Zemke *et al*. 2002; Letard *et al*. 2008; Vozdova *et al*. 2019). Additionally, it has been shown that patients with c-KIT mutations are associated with a higher grade of MCT (Zemke *et al*. 2002) and have an increased risk of recurrence as well as significantly increased mortality due to MCT-related disease (Webster *et al*. 2006).

Apart from MCT in dermis and subcutaneous tissue, the tumor can also occur extracutaneous, for instance in the gastrointestinal tract, conjunctiva, oral cavity and urethra (Welle *et al*. 2008). Further, a visceral form of MCT exists, known as systemic mastocytosis. Systemic mastocytosis, which usually is preceded by a primary cutaneous tumor, affects the local lymph nodes, liver, spleen, and bone marrow. Systemic mastocytosis is a more aggressive variant, coherent with poor prognosis. The cutaneous MCT can be divided into well-differentiated or non-differentiated, where the latter can spread and become systemic mastocytosis. The non-differentiated and more aggressive MCT is coherent with decreased survival time whereas the well-differentiated and less aggressive has a good survival prognosis (Patnaik *et al.* 1984).

Cutaneous MCT has a heterogenic appearance, mostly depending on its malignancy (London & Thamm 2013). Well-differentiated are often solitary, small, slow growing without ulceration, and can be mistaken for a non-neoplastic change. On the other hand, the non-differentiated MCT form tend to be fast-growing, ulcerated with inflamed surrounding tissue. Manipulation and palpation of the tumor can result in degranulation followed by histamine-triggered erythema of surrounding tissue, a clinical condition called Darier´s sign, which also can appear spontaneously. Common locations of the skin tumor includes extremities, neck, thorax and abdomen (Welle *et al.* 2008; Thompson *et al*. 2011).

Gastrointestinal ulceration due to MCT can be seen, as well as other symptoms such as anorexia, abdominal pain, vomiting and melaena (O'Keefe *et al*. 1987; Welle *et al*. 2008). These symptoms are usually seen in aggressive MCT and the visceral form. The ulceration is caused by an elevated level of histamine in the blood stimulating the H2-receptor on parietal cells leading to excessive production of gastric acid (de Nardi *et al*. 2022).

Canine MCT is diagnosed through a fine-needle cytology (de Nardi *et al*. 2022). A tissue sample is collected with a fine needle, with or without aspiration. The sample is then dyed and evaluated in a microscope. The method is non-invasive, quick, cheap, and accurate. The tumor is graded based on histopathological features of a biopsy. One commonly used grading system for cutaneous MCT is the Patnaik grading system. This grading system divides the tumors into three groups: 1, 2 and 3 (Patnaik *et al*. 1984). Grade 1 is characterized by rows or small groups of well-differentiated, monomorphic, round mast cells in between of the dermal tissue. The MCT contain no mitotic cells and have minimal necrosis or edema. Grade 2 is distinguished by groups of moderately pleomorphic cells infiltrating dermal and subcutaneous tissue or even deeper tissue. A few mitotic cells may exist, from 0 to 2 per high power field. The tumor cells are round to oval with, in some cases, indistinct cytoplasm and hyperchromatic granules. Areas with diffuse edema and necrosis may be present. The most malignant group, grade 3, is characterized by pleomorphic cells in highly cellular and dense sheets replacing the normal subcutaneous and deeper tissue. The neoplastic cells are round, oval, or spindle-shaped with indistinct cytoplasm. Double nuclei are frequently seen as well as mitotic cells, ranging from 3 to 6 per high power field. Areas of edema and necrosis are common. Later, a two-group based grading system was proposed by Kiupel *et al*, dividing MCT into low grade and high grade (Kiupel *et al*. 2011). The criteria for high grade MCT are the presence of one or more of the following criteria: ≥7 mitotic cells in 10 high power fields, ≥3 cells with 3 or more nuclei in 10 high power fields, ≥3 atypical nuclei with marked indentations, segmentations, and irregular shape in 10 high power fields and/or karyomegaly.

2.3 Serglycin

Serglycin is a proteoglycan constructed of a core protein with negatively charged glycosaminoglycans (GAGs) attached (Kolset & Tveit 2008). Heparin, sulfated chondroitin and heparan sulfate are different types of GAGs usually associated with serglycin. Serglycin is categorized as an intracellular proteoglycan, although it can also be secreted into the extracellular matrix and attached to the cell surface (Schick *et al*. 2001; Kolset & Tveit 2008). Serglycin is extensively expressed by hematopoietic cells, particularly in mast cells (Tantravahi *et al*. 1986), but also non-hematopoietic cells such as endothelial cells (Schick *et al*. 2001), smooth muscle cells (Kulseth *et al*. 1999) and chondrocytes (Zhang *et al*. 2010), among other. The proteoglycan is also expressed by several tumor cells, where the level of expression corresponds to the malignancy of the cancer type (Korpetinou *et al*. 2015).

A single gene encodes for the peptide core of serglycin and is located on chromosome four, 20.503.157-20.517.696, in dogs and consists of three exons

(NCBI n.d.). The peptide core consists of 158 amino acids with tandem repeats of serine and glycine (Tantravahi *et al*. 1986; Korpetinou *et al*. 2014). The function of the serine-glycine repeats is attachment site for the GAGs (Bourdon *et al*. 1985).

Several studies have shown that serglycin is essential for storage of various components in secretory granules in different cells, such as connective tissue-type mast cells, mucosal mast cells, cytotoxic T lymphocytes and neutrophils (Åbrink *et al*. 2004; Grujic *et al*. 2005; Braga *et al*. 2007; Niemann *et al*. 2007). In mast cells one function of serglycin is storage and release of inflammatory mediators in intracellular granules, such as dopamine (Rönnberg *et al*. 2012), serotonin and histamine (Ringvall *et al*. 2008). Thereby, the proteoglycan serglycin has an important role in inflammatory reactions (Kolset & Tveit 2008; Roy *et al*. 2016; Ahrens *et al*. 2020).

In a study on serglycin knockout mice, it was shown that the mast cells were gravely affected by the absence of serglycin (Åbrink *et al*. 2004). The mast cells had different morphology and decreased ability to store proteases in granules. The granules in the mast cells from serglycin deficient mice appeared to be empty during maturation, suggesting that serglycin is essential for maturation of the granules and storage of proteases normally found in mast cells. Similar lack of storage was shown in the mature granules of cytotoxic T lymphocytes from serglycin deficient mice (Grujic *et al*. 2005). The absence of serglycin in these cells also resulted in granules deficient of granzyme B, an enzyme involved in inducing apoptosis in target cells. This suggests that serglycin is essential for storage of granzyme B. The neutrophils from serglycin deficient mice were not affected at a structural level in contrast to the mast cells (Niemann *et al*. 2007). However, serglycin-deficient neutrophils lacked elastase in azurophilic granules, a protease involved in inflammation (Korkmaz *et al*. 2008), which suggests that serglycin is necessary for elastase to be localized in these granules. Further, when injecting the mice with *Klebsiella pneumoniae* it was revealed that the virulence was increased in the serglycin deficient mice compared to the regular mice (Niemann *et al*. 2007). This points to the fact that lack of serglycin negatively impacts the immune system.

In a study from 1995 it was found that serglycin with sulfated chondroitin is a ligand for the cell adhesion molecule CD44 (Toyama-Sorimachi *et al*. 1995). CD44 is active in cell-to-cell interactions in immune reactions, such as activation of lymphocytes. The binding of serglycin to CD44 can stimulate leukocyte adhesion and activation of cytotoxic T-lymphocytes. An increased expression of serglycin was seen in endothelial cells after stimulation by the proinflammatory cytokine TNF-alfa (Kulseth *et al*. 1999). This indicates that serglycin is involved in leukocyte migration in inflammatory processes.

2.3.1 Serglycin and cancer

In several studies it has been reported that aggressive cancer cells have a higher expression of serglycin and that high levels of the proteoglycan therefore is associated with poor prognosis (Korpetinou *et al*. 2015; Roy *et al*. 2016; Ahrens *et al*. 2020).

In a study by Roy *et al*. (2017) about serglycin as a potential biomarker for glioma it was shown that serglycin can promote aggressiveness in glioma and is also positively correlated to more malignant forms of glioma as well as low survival rate. In a study on mammary tumors in mice, the mice with serglycin-deficient tumors showed no sign of lung metastasis unlike the tumors able to express serglycin (Roy *et al*. 2016). This suggests that serglycin is necessary for metastasis in mammary tumors. However, the growth of the primary tumor was not affected by the absence of serglycin. Similar attributes of serglycin, as described above, has been shown in other cancer types, such as non-small cell lung cancer (Guo *et al*. 2017), nasopharyngeal carcinoma (Li *et al*. 2011), liver cancer (He *et al*. 2013) and breast cancer (Korpetinou *et al*. 2013).

As earlier mentioned, serglycin can bind to CD44 which the proteoglycan also does in the tumor microenvironment (Korpetinou *et al*. 2014). This adhesion molecule, CD44, promotes migration, metastasis, epithelial to mesenchymal transition (EMT), and apoptosis resistance in cancer (Zöller 2011). EMT is a process where epithelial cells undergo biochemical changes and progress to mesenchymal cells (Li *et al*. 2011). The cell loses its adhesion to matrix and to other cells which enhances migratory and invasive qualities. The process is essential in embryotic development but is also involved in cancer metastasis and progression.

2.4 CRISPR/Cas9

CRISPR/Cas9 is a method used to make specific changes in the genome. The method was developed by Jennifer Doudna and Emmanuelle Charpentier, who were awarded with the Nobel prize in chemistry 2020. CRISPR/Cas9 was developped from a natural defense system used by bacteria to defend themselves against phage infection and plasmid transfer (Barrangou *et al*. 2007). The CRISPR-associated enzyme, or Cas9, is a DNA endonuclease that binds to the target DNA-sequence using a guide RNA and thereafter makes a double strand break (DSB) in the DNA (Jinek *et al*. 2012). A sequence motif of 2-5 base pairs, called PAM, located on the complementary strand close to the gRNA-target sequence is of importance for the Cas enzyme to be able to bind to the DNA helix. The PAM sequence for Cas9 consists of “NGG” where “N” could be any nucleotide base

followed by to guanine bases. Cas9 cleaves the DNA 3 base pairs upstream of the PAM.

When Cas9 makes a DSB, the DNA is repaired with non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Cong *et al*. 2013). NHEJ often results in removal or addition of one to hundreds of nucleotides and can cause a frame-shift mutation which disrupts the gene expression and in extension its function (Lieber 2008). The DSB can be repaired through HDR when an exogenous DNA molecule, with 5´ and 3´ ends that have an identical nucleotide sequence to the area around the DSB, is present. The homologous DNA is then used as a repair template. This DNA molecule can also contain an exogenous gene that the cell starts to express after insertion (Dow 2015). This technology makes genome editing possible by inactivating genes and thereby explore their function.

3. Material and methods

3.1 Cell line

The canine MCT cell line, C2, established by Prof. Warren Gold year 1990 (DeVinney & Gold 1990), was obtained from Prof. Einspanier lab in Berlin and with a permissive use from Prof. Gold.

3.2 Methods

The methods used in this study were cell culture, transfection with CRISPR/Cas9 using electroporation, and genomic PCR. Genomic PCR was made on the C2 extracted DNA to control the serglycin sequence of the exons and if it corresponds to the standard genomic organization, and to test the screening design for detection of the HDR inserted selection cassette.

3.2.1 Cell culture

The tumor cells were cultured in suspension in RPMI 1640 medium, in 37 degrees Celsius and 5% carbon dioxide. The medium was supplemented with 10% fetal bovine serum. To prevent bacterial growth, 1% Sodium Pyruvate (5ml of 100mM) and 1% penicillin-streptomycin (5ml of 10 000U/ml, 10mg/ml) were added to the medium. Cell culture flasks (T-25 and T-75) were used containing 5 respectively 15 ml medium. After 3-4 days, when the cells had increased in number, they were moved to new flasks or partly discarded. When changing the medium or divided into new flasks the medium with cells were poured into a falcon tube and centri-fugated at 1100 revolutions per minute for 5 min. The supernatant was poured out and the pellet of cells in the bottom of the falcon tube was diluted with medium and put into one or two cell culture flasks.

3.2.2 Transfection with CRISPR/Cas9

The gRNA was designed with CRISPOR, a program from tefor Paris-Saclay, with data from “NCBI gene” (https://www.ncbi.nlm.nih.gov/gene/?term=) by Sofia

Tengstrand (Table 1). The transfection site in C2 was located at exon 2 of the serglycin gene.

Table 1. The gRNA used for transfection.

	C2 (SRGN exon 2)
gRNA 5'→3' (PAM)	GCAGTGTACCCACTGGTACG *(TGG)*

The double strand break (DSB) by CRISPR/Cas9 was repaired with HDR (homology directed repair) using two versions of a cassette containing genes for either green fluorescence protein and resistance to the antibiotic puromycin or red fluorescence protein and resistance to the antibiotic blasticidin. The cassette also contained an exogenous promotor for the fluorescence gene and three stop codons at the end of the cassette (Figure 1). Homology arms, with matching sequences upstream and downstream from the DSB, was used to guide the gene cassette to be inserted at the right location. The primers shown in Figure 1 were used in PCR reactions when confirming the presence of the gene cassette in the transfected cells.

Figure 1. The structure of the gene cassette. The target gene is the homology arms matching the sequence upstream and downstream from the DSB. The 2A is a self-cleaving peptide linked to Puro/Blast. Puro/Blast are genes for resistance to the antibiotics puromycin respectively blasticidin. CMV is an exogenous promotor that drives the expression of GFP/RFP. GFP stands for green fluorescence protein and RFP for red fluorescence protein. 3X Stop are stop codons for termination of the protein translation in three different reading frames. F1 and R1 are the forward resp. reversed primers in the SRGN gene, presented in table 4. F2 and R2 are the forward resp. reversed primers in the Puro/Blast gene. F3 and R3 are the forward resp. reversed primers in the GFP/RFP gene.

Electroporation

A pilot study was made to decide the optimal voltage setting of the electroporation, where the cell membrane become permeable due to a short electrical pulse. The attempt was performed using a gRNA in exon 3 and a GFP marked Cas9-enzyme. Three different settings were chosen: 200V, 250V and 300V. The capacitance was set to 500 µF. One negative control, without Cas9, and one positive sample, containing Cas9, for each voltage setting was electroporated, resulting in a total number of six samples. One day after electroporation, the cells were observed in a fluorescence microscope, revealing that many cells were fluorescent, indicating the successful entry of the GFP-marked Cas9 enzyme into the cells. Four days after the electroporation the cells were counted with trypan blue, making the dead cells blue, in a Fuchs-Rosenthal chamber to see what percentage of cells had died. This resulted in 250V as the optimal voltage with a cell mortality of approximately 85%.

Subsequently, the transfection with CRISPR/Cas9 using electroporation was performed at 250V (Appendix 1). Firstly, cells were washed with PBS (phosphate-buffered saline). Secondly, the number of cells were counted in a Fuchs-Rosenthal chamber. The average amount of cells, in three group squares, were multiplied with a factor 5000 to receive the number of cells per millilitres. The electroporation was also made with one negative control without Cas9, gRNA, or the gene cassette. 1-1,5 million cells were collected per reaction, hence, about 3 million cells were used in total.

Selection with antibiotics

Following electroporation, the cells were cultured in pre-heated RPMI-medium for one day. On the subsequent day, antibiotic selection was initiated to isolate the cells in which the transfection had been successful. The cells were cultured in a 6 well-plate. The transfected cells were divided into five, one positive control without antibiotics and four wells with different antibiotic concentration (Table 2). The last well contained the negative control, cells electroporated without Cas9. The different concentrations were based on the information of the range of each antibiotic, that cells are normally susceptible to, from ThermoFisher (https://www.thermofisher.com/se/en/home/life-science/cell-culture/transfection/transfection-reagents/selection-antibiotics.html).

The medium was initially changed once every day for three days, thereafter every second day for six days followed by one medium change four days later. The antibiotic selection was retained for 13 days in total.

Table 2. Concentrations of puromycin and blasticidin in medium A-D.

	Puromycin (µg/ml)	**Blasticidin (µg/ml)**
Medium A	0.5	1
Medium B	1	2
Medium C	2	5
Medium D	5	10

To estimate cell mortality after the antibiotic selection, a fraction of the cells was dyed with trypan blue. The cells were then counted in a Fuchs-Rosenthal chamber.

3.2.3 Analysis with genomic PCR

A genomic PCR of the sequence of serglycin in the tumor cells was made according to the protocol for "PCRBIO Rapid Extract PCR Kit" (Appendix 2). This was done by lysis, nuclease, and protein denaturation, followed by PCR reaction. The primers were designed with NCBI Primer-BLAST tool, choosing primers with the length

of approximately 25 base pairs, containing 40-60% cytosine and guanine, ending with at least one cytosine or guanine at the 3' end and preferably not having three or more cytosines or guanines in a row (Table 4).

Table 4. The primers used for genomic PCR.

	Forward primer (5'→3')	**Reversed primer** (5'→3')	**PCR product size**
C2, SRGN exon 2	TTCCTGTTTGGACAGGTTCTCCTG	CTGGAAGTAGGTCAAACATTGGTTC	120 base pairs

The temperature of annealing of the designed primers were 58.8ºC and 55.9ºC for the forward respectively reversed primer. The PCR reaction setup was made with half of the volumes in the protocol. One negative control, without DNA sample, for each pair of primer was analyzed. The PCR reaction was set at 95ºC for 5 min, followed by 35 cycles of 95ºC for 30 seconds (denaturation), 60ºC for 30 seconds (annealing) and 72ºC for 1 minute (extension). After that, 10 minutes at 72ºC and 4ºC for about 10 minutes.

Gel electrophoresis

The products of the PCR were analyzed on an agarose gel electrophoresis. The gel was made of 1.5% agarose boiled in a TAE-solution. The fluorescent nucleic acid dye "GelRed® Nucleic Acid Gel Stain" was added to the PCR-products and they were run on the gel in TAE-solution at 100 V for approximately 30 min. The result was observed in ultraviolet light.

4. Results

4.1 Transfection with CRISPR/Cas9

The expression of the fluorescence gene was verified in a fluorescence microscope. Almost every cell in the wells was releasing a green fluorescent light, even in the negative control (Figure 2). Multiple cells also emitted a faint red fluorescent light (Figure 3). The cells were also observed in a fluorescence microscope after mounted on an object glass. On the object glass the cells had a bright red and green fluorescent light, even in the negative control (Figures 4 and 5).

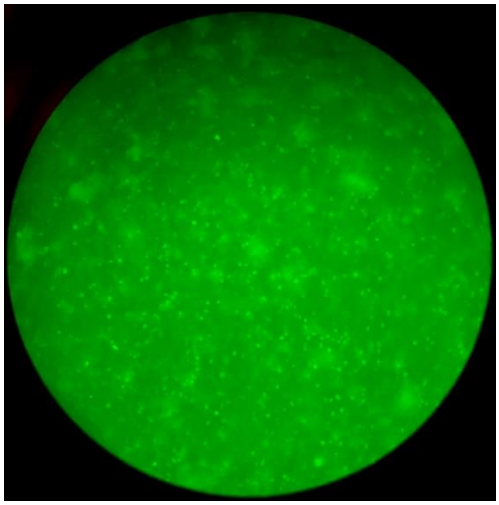

Figure 2. Cells in the well of the negative control inspected in green fluorescent light.

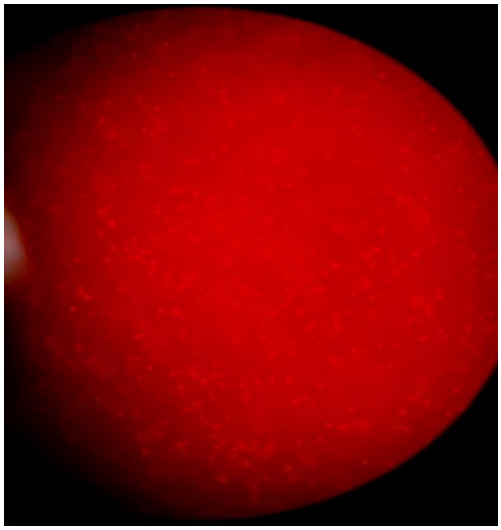

Figure 3. Cells in the well of the negative control inspected in red fluorescent light.

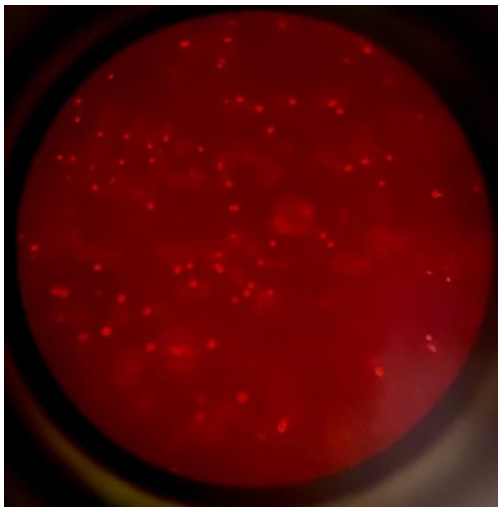

Figure 4. Cells from the negative control mounted on an object glass in red fluorescent light.

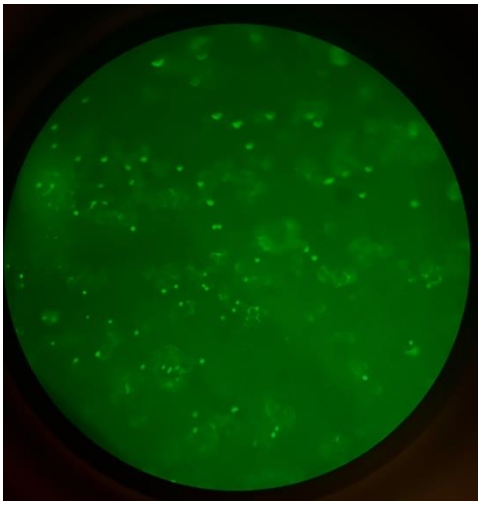

Figure 5. Cells from the negative control mounted on an object glass in green fluorescent light.

Selection with antibiotics

After 13 days the selection with antibiotics was terminated. The estimated cell mortality 17 days after the electroporation was 15% in the negative control, 24% in the positive control and up to 100% in well A, B, C and D.

4.2 Molecular analysis

4.2.1 Genomic PCR

Before transfection

To verify the designed primers in table 4, a PCR reaction was set up. The reaction was performed using the wild-type C2 cells. The primers resulted in one band with a size of approximately 100 bp (Figure 6).

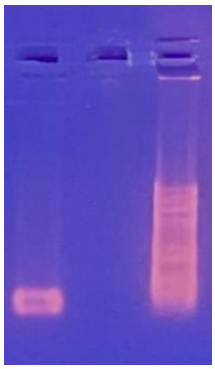

Figure 6. Results from the PCR with SRGN forward and reversed primers. Lane 1: Primers and genetic DNA material from C2. Lane 2: Negative control with only primers. Lane 3: Ladder (50-1500 bp)

After transfection

One PCR reaction to confirm the gene cassette in the transfected cells was done with SRGN forward primer (F1 in Figure 1) and TrueTag GFP reversed primer (R3 in Figure 1). The genomic DNA material used was pooled from the transfected cells in the wells with and without antibiotic selection. The results showed two bands in lane 1 with the size of approximately 300 bp and 250 bp and one band in lane 2 with the size of 300 bp (Figure 7).

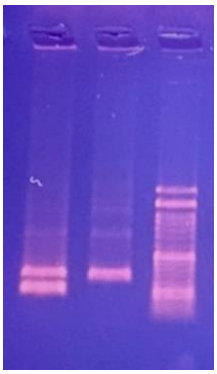

Figure 7. Confirmation of the gene cassette in the transfected cells, using SRGN forward primer and TrueTag GFP reversed primer. Lane 1: Primers and pooled genomic DNA. Lane 2: Negative control with only primers. Lane 3: Ladder (50-1500 bp).

Confirmation of correct insertion of the gene cassette in each one of the wells with antibiotic and the control without antibiotics was then performed. This resulted in two bands in both lanes, one with the size of approximately 300 bp and one of 250 bp (Figure 8), identical to the result of the pooled DNA material (Figure 7).

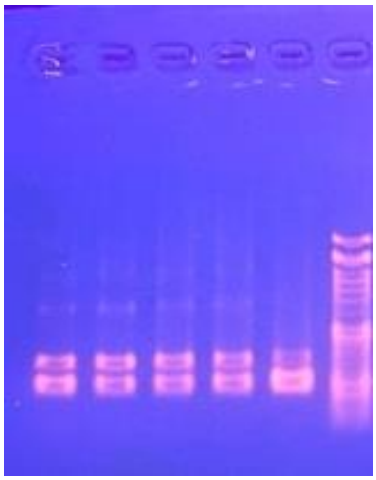

Figure 8. Confirmation of the gene cassette in the transfected cells after the antibiotic selection, using SRGN forward primer and TrueTag GFP reversed primer. Lane 1: Primers and genomic DNA from medium A. Lane 2: Primers and genomic DNA from medium B. Lane 3: Primers and genomic DNA from medium C. Lane 4: Primers and genomic DNA from medium D. Lane 5: Primers and genomic DNA from medium without antibiotics. Lane 6: Ladder (50-1500 bp).

The second confirmation of the gene cassette was performed on genomic DNA from each well with and without antibiotics with SRGN forward primer (F1 in Figure 1) and TrueTag RFP reversed primer (R3 in Figure 1). The result showed one clear bright band in every lane with the same size of approximately 300 bp (Figure 9).

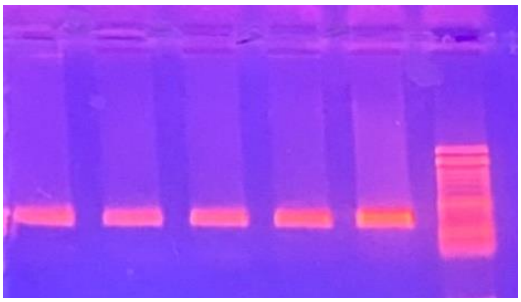

Figure 9. Confirmation of the gene cassette in the transfected cells after the antibiotic selection, using SRGN forward primer and TrueTag RFP reversed primer. Lane 1: Primers and genomic DNA from medium A. Lane 2: Primers and genomic DNA from medium B. Lane 3: Primers and genomic DNA from medium C. Lane 4: Primers and genomic DNA from medium D. Lane 5: Primers and genomic DNA from medium without antibiotics. Lane 6: Ladder (50-1500 bp).

Another PCR was performed on genomic DNA from the wells with and without antibiotics with TrueTag RFP forward primer (F3 in Figure 1) and SRGN reversed primer (R1 in Figure 1). A faint band in the first lane was shown with the size of approximately 150 bp (Figure 10).

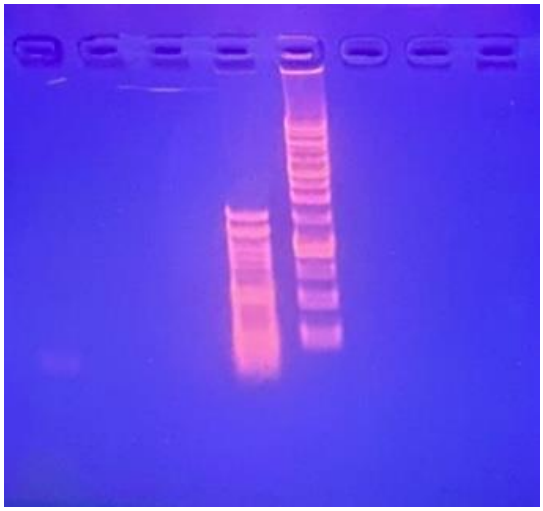

Figure 10. Confirmation of the gene cassette in the transfected cells after the antibiotic selection, using TrueTag RFP forward primer and SRGN reversed primer. Lane 1: Primers and genomic DNA from medium A. Lane 2: Primers and genomic DNA from medium B. Lane 3: Primers and genomic DNA from medium C. Lane 4: Ladder (50-1500 bp). Lane 5: Ladder (250-10000 bp). Lane 6: Primers and genomic DNA from medium D. Lane 7: Primers and genomic DNA from medium without antibiotics. Lane 8: Negative control with only primers.

No products were seen on the gel electrophoresis when a PCR reaction was done with TrueTag GFP forward primer (F3 in Figure 1) and SRGN reversed primer (R1 in Figure 1). The same PCR reaction was performed with a lower annealing temperature, since the reversed primer had a temperature of 55,9^{o}C, but without improvement. The PCR was done again with another SRGN reversed primer without any products.

PCR for confirmation of the gene cassette in the transfected cells using the homology arms as primers was also performed. One reaction was done on pooled genomic material, one positive control with homology arms and the cassette, and one negative control with only the homology arms. In all reactions a product with a size shorter than 250 bp was detected (Figure 11). A second product in the positive control was present with a size of 2500 bp.

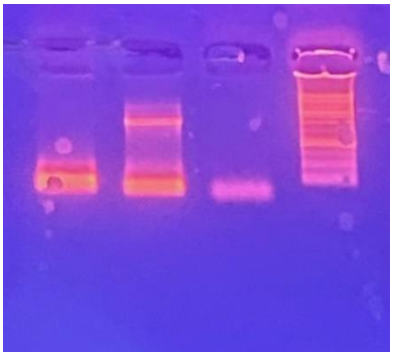

Figure 11. Confirmation of the gene cassette in the transfected cells after antibiotics selection using the homology arms as primers. Lane 1: Homology arms and pooled genomic DNA. Lane 2: Positive control with the homology arms and the gene cassette. Lane 3: Negative control with the homology arms. Lane 4: Ladder (250-10000 bp).

5. Discussion

The purpose of this study was to develop a serglycin deficient canine mastocytoma cell line, using CRISPR/Cas9. After this study, the future goal is to explore changes in morphology, growth, and metastasis, but also to identify which proteins and mediators that have been lost or changed in comparison to the wild type. This information can increase the understanding of serglycins role in the progress of cancer.

5.1 Cell culture

The cell culture was successful throughout the study, without any infection in the cell culture flasks. When the cells grew in an excessive amount in proportion to the medium volume, they started to lump together. The cells grew faster in the close presence of other cells compared to if the cells were further apart. After two months in the same cell culture flask the cells began to grow adherent to the bottom of the cell culture flask and the morphology changed to a more oblong appearance. After the electroporation most of the cells became pyknotic, small and dense which indicated that they were dead.

5.2 Transfection with CRISPR/Cas9

After the transfection with CRISPR/Cas9 and insertion of the gene cassette, the cells were examined in a fluorescence microscope. A fraction of the cells was mounted on object glass with VectaMount and examined again. The cells in the wells and on the object glasses beamed with both green and red light, both in the control (Figures 2, 3, 4 and 5) and the transfected cells. This indicated that the cells were autofluorescent in both green and red. The cells being autofluorescent in green and red made it impossible to subclone the successfully transfected cells based on fluorescence. It was no longer possible to perform fluorescence-activated cell sorting (FACS) after this discovery.

The selection with antibiotics was initiated the day after electroporation based on the transfection protocol. Many cells died in the wells containing antibiotics in the first days and 17 days after the electroporation the mortality was up to 100% in the

well containing the lowest concentration of antibiotics. The living cells had a hard time to recover and multiply compared to the well without antibiotics. A possible factor contributing to the difficulty multiplying and surviving is that the cells were further apart due to the low number of cells after a few days in antibiotic selection. The reason, as previously mentioned, is that the cells grow faster in the close presence of other cells. The high mortality also indicates that the selection might have started too soon after the electroporation or that the concentration of antibiotics was too high.

To confirm the appropriate concentrations of antibiotics, establishing a kill curve would be of advantage. A kill curve determines the minimum concentration of an antibiotic that is lethal for the wild-type cells. With that information the chosen concentrations for the selection would have been confirmed to be better suited for this cell line. The selection with antibiotics could favorably have been initiated a couple of days later to give the cells an opportunity to recover properly after the electroporation. Since the medium contained both puromycin and blasticidin, the selection only enabled further living of the cells that had incorporated both versions of the gene cassettes in both alleles to express resistance to both puromycin and blasticidin. It is a possibility that no cell had integrated both gene cassettes, and that several cells had incorporated one version of the cassette in both alleles and therefore only expressed resistance against one of the antibiotics.

5.3 Molecular analysis

5.3.1 Genomic PCR

The verification of the primers in the SRGN gene of the wild-type C2 (Table 4) resulted in a single band with a size of approximately 100 bp (Figure 6), which matched the expected product of 120 bp. No band was seen in the negative control with only primers, confirming no primer dimers had been produced. This strongly indicates that the primers were working.

The first PCR reaction to confirm the gene cassette in the transfected cells was amplifying the product from SRGN forward primer to the TrueTag GFP reversed primer. This PCR resulted in two distinct bands in the lane containing DNA material from transfected cells and one band in the negative control, which was the same size as the longer product in the first lane (Figure 7). The shorter product in the first lane suggests that the gene cassette has been incorporated in the right place. A product with the same size as in the negative control was also seen in the wells in Figure 8. This band could have been a primer dimer or the primers was able to bind to a different location in the genome and to amplify a product of 300 bp. If

being the latter, it also means that the SRGN forward primer could bind upstream close to the location of GFP reversed primer to produce a product with the size of appr. 300 bp. This being less likely than a primer dimer. However, a primer dimer of 300 bp suggests that several primers bound to each other to build this product since the primers are 24 resp. 22 bp long.

Another PCR to control the presence of the gene cassette was set up with SRGN forward primer and TrueTag RFP reversed primer. The clear bright bands of the same sizes indicated that there were similar products in every well (Figure 9). Hence, it is a proof of the gene cassette being incorporated in the right place in the genome in some cells in every well.

To verify the gene cassette on the 3´ side, a PCR reaction with TrueTag RFP forward primer and SRGN reversed primer was made. A faint band in the first lane from medium A was shown, with the size of approximately 150 bp (Figure 10). Together with the product from SRGN forward primer and TrueTag RFP reversed primer, this could indicate that the whole cassette was present in a few cells in the well with medium A. However, it is also possible that the product was a primer dimer. On the other hand, the product should have existed in every lane if it was a primer dimer.

Furthermore, no products were seen in the corresponding PCR reaction with TrueTag GFP forward primer and SRGN reversed primer, even after adjustments of the annealing temperature and using another reversed primer. This result indicates that the reaction needs to be optimized or that the 3´ end of the cassette was not present. The latter lowers the possibility of the product in the former PCR reaction (Figure 10) to be the gene cassette since if the RFP version is present in some cells, the GFP version should be too.

Since all lanes except one were blank in the verification of the gene cassette on the 3´ side (Figure 10), a PCR reaction was set up with the homology arms as primers. In all lanes a product shorter than 250 bp was produced (Figure 11). This product was too short to be the gene cassette, which was approximately 2400 bp, and more likely to be a primer dimer since it was also present in the negative control. In the positive control a larger product of about 2500 bp was visible. This product was most likely the gene cassette, which means that the homology arms could bind to the cassette correctly. However, this large product was not present in the pooled DNA material from the transfected cells. This means that the gene cassette was not incorporated in the cells, at least not as a whole.

5.4 Conclusion and future development of the study

In conclusion, the PCR reactions set up to confirm the presence of the gene cassette at the right location in the genome, showed products from SRGN forward primer to GFP and RFP reversed primers. However, no clear product was seen on the 3´ side. It is possible that the gene cassette had fractured downstream of the GFP and RFP reversed primers. Another explanation is that the primers made products from locations in the genome other than at the serglycin gene. The possibility of this is extremely low since all three primers then should be able to bind to another location in the genome all close to each other.

Further, to give the cells an opportunity to recover after the electroporation the selection with antibiotics could have been initiated at day four of the experiment instead of at day two. Delaying the selection with antibiotics two days would still be according to the protocol. In addition, establishing a kill curve of the two antibiotics for the C2 cell line prior the transfection would be an advantage.

Due to delay in the delivery of some material essential for the transfection, some analyzes were not carried out as initially planned, for example subcloning and quantitative polymerase chain reaction (qPCR). Hence, the next step following this study is to subclone the cells to investigate if the knockout of the serglycin gene has been successful in any cell. This can be done by limiting dilution, distributing the cells in a 96-well plate, to obtain one cell in every well. The isolated cells will multiply to generate clones for subsequent analysis. Another possible analysis is FACS (fluorescence-activated cell sorting), a machine method to separate successfully GFP-and RFP-transfected cells from non-transfected. However, since the C2-cells were found to be autofluorescent it is not certain that this will work. After succeeding with the subcloning process, it is necessary to conduct a qPCR to measure the expression of serglycin in the transfected cells. When a serglycin deficient mastocytoma cell line has been established, future analyzes will study the changes in morphology, growth, and metastasis to explore the role of serglycin in carcinogenesis.

The antibiotics used in this study were autoclaved and destructed in a special container. There were no animal ethics or other environmental aspects in this project to consider.

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Popular science summary

Serglycin is a molecule produced by several cell types and by cancer cells. It has been shown that a high expression of serglycin is associated with more aggressive cancer types and therefore a poorer prognosis. Further, several types of cancers have been reported to be dependent on serglycin for its ability to spread in the body.

Mastocytoma, or mast cell tumor (MCT), is a tumor originating from a blood cell involved in the immune system called mast cell. Mast cells are found in every organ throughout the body, with a large amount in the skin, stomach, and intestines. Hence, MCT often occur at these locations, especially in the skin. MCT is one of the most common skin tumors in dogs and affects mostly older individuals. MCT in the skin have a wide range in their appearance. They can be small lesions mistaken for a non-neoplastic change, a solitary slow growing lump, or fast-growing lesions with inflamed surrounding tissue.

In the present study, CRISPR/Cas9 was used to cut in the gene of serglycin to develop serglycin deficient MCT cell lines from canine. After this study, the future goal is to explore changes in growth, and ability to spread in the body in comparison to the normal MCT cell type. The purpose is to increase our understanding of the role of serglycin in the progress of cancer.

The methods used were cell culture, CRISPR/Cas9 to insert a gene cassette in the cells using an electrical pulse, and genomic polymerase chain reaction (PCR) to verify if the method was successful. The gene cassette contained genes for resistance against two types of antibiotics (puromycin and blasticidin) and fluorescent color to make the cells glow in green and red fluorescent light. Thereafter, the cells were treated with antibiotics to isolate the cells with the resistance gene. A fluorescence microscope was used to find cells with the fluorescent light gene.

The modified cells were observed in a fluorescence microscope. Almost every cell in the wells was releasing a green fluorescent light and multiple cells also emitted a faint red fluorescent light, even in the negative control. The cells were also observed in a fluorescence microscope after mounted on an object glass. On the object glass the cells had a bright red and green fluorescent light, even in the

negative control. This indicates that the cells were glowing in both green and red without the gene from the gene cassette. After 13 days of antibiotic treatment up to 100% of the cells had died. The reason for this could be that the antibiotics were added too soon after the electroporation since this has a severe impact on the cells. It is also possible that the concentration of the antibiotics were too high.

PCR reactions were set up to verify the presence of the gene cassette in the cells. The results showed that the first part of the gene cassette was present in the right location in the genome of the cells. This means that the gene for antibiotic resistance is present in the cells. However, there were no results when verifying the last part of the gene cassette. This could mean that the gene cassette fractured in one of the steps of the methods.

The conclusion of this study is that further analysis, for example subcloning and qPCR, is necessary to determine if the knockout of the serglycin gene has been successful in the cells.

Acknowledgements

I would like to express my gratitude to my supervisor, Magnus, for his invaluable support, dedication, and expertise throughout this study. I am also grateful to my assistant supervisor, Sofia, for the guidance and constructive feedback contributed to the refinement of this thesis. A special thanks to my fellow student, Mattias, for his collaborative insights in the laboratory and support.

Appendix 1: Transfection protocol

Preparations (performed days before transfection)

Prepare working stock of gRNA solution:

Information from TrueGuide™ Synthetic Guide RNA User Guide (Pub. No. MAN0017058 Rev. D.0) (thermofisher.com)

Resuspend TrueGuide™ Synthetic gRNA in TE buffer to prepare 100 µM (100 pmol/µL) stock solutions using this procedure.

1. Before opening, centrifuge each TrueGuide™ Synthetic gRNA tube at low speed (maximum RCF 4,000 × g) to collect the contents at the bottom of the tube, then remove the cap from the tube carefully.
2. Using a pipette and sterile tips, add the required volume of TE buffer to prepare 100 µM (100 pmol/µL) stock solutions. For 1.5 nmol of sgRNA, this volume equals 15 µL and for 3 nmol, this volume equals 30 µL.
3. Vortex the tube to resuspend the oligos, briefly centrifuge to collect the contents at the bottom of the tube, then incubate at room temperature for 15–30 minutes to allow the sgRNA oligos to dissolve.
4. Vortex the tube again to ensure that all the contents of the tube are resuspended, then briefly centrifuge to collect the contents at the bottom of the tube.
5. Optional: Check the concentration of the resuspended oligos using the NanoDrop™ Spectrophotometer (or equivalent) or a UV-base plate reader.
6. Optional: Create working stock of 1 µM solution: add 99 µL TE Buffer to 1 µL of the 100 µM solution in a new tube. Follow the vortex and centrifuge steps above for mixing.
7. Use working stocks immediately or freeze at –20°C until use

Create donor DNA:

Information from TrueGuide™ Synthetic Guide RNA User Guide (Pub. No. MAN0017058 Rev. D.0) (thermofisher.com)

"PCR is done using the custom-designed homology arm primers, the donor template, and the included Phusion™ Flash High Fidelity PCR Master Mix. The Phusion™ master mix has very high fidelity to minimize the chance of PCR introduced errors. The template is provided in a linear format for more robust PCR amplification."

This protocol will generate >5µg of linear dsDNA donor with the desired gene specific homology arms. Our experiment uses GFP+Puro and RFP+Blast combinations.

1. Set up two 50-µL PCR reactions:

Component	Amount
2X Phusion™ Flash High Fidelity PCR Master Mix	25 µL
Forward primer (10 µM)	1 µL
Reverse primer (10 µM)	1 µL
Donor template (20 ng/µL)	1 µL
Water	22 µL
Total	50 µL

2. PCR parameters:

Step	Cycles	Temperature	Time
Denature	1	98°C	10 seconds
Amplification	30	98°C	1 second
		70°C	5 seconds
		72°C	10-30 seconds/kb
Final extension	1	72°C	2 minutes
Final hold	1	4°C	HOLD

3. Confirm successful PCR reaction by running 2 µL of PCR on 1–2% agarose gel. A successful PCR will have the following band sizes:

TrueTag™ DNA donor type	Band Size (kb)
Fluorescent	1.3–1.5
Epitope	0.5–0.9
Stem	1.4–1.8
Knockout Enrichment	2.1–2.4

Note: The adaptor primers for PCR amplification are ~56 bases, so occasionally the PCR reaction may fail. If this happens, breaking down the forward and reverse adaptor primers into two 21-nt overlapping inner and outer forward primers and two 21-nt inner and outer overlapping reverse primers will generally resolve this issue. See example in manual.

➔ Continue on next page

4. PCR Cleanup using the TrueTag™ PCR Purification module. All steps should be carried out at room temperature. All centrifugations should be carried out in a table-top microcentrifuge at >12,000 × g (10,000–14,000 rpm, depending on the rotor type).
    - Prior to the initial use of the purification module, dilute the Wash Buffer (concentrated) with 9 mL of ethanol (96–100%)
    - Examine the Binding Buffer for precipitate before each use. Re-dissolve any precipitate by warming the solution to 37°C and cooling to 25°C.
    a. Combine the two completed 50-µL PCR reactions for *each donor* into 1 tube
    b. Add a 100 µL of Binding Buffer to completed PCR mixture (1:1). Mix thoroughly. Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.
    c. Transfer the 200 µL of the solution from step b to the GeneJET™ purification column. Centrifuge for 30–60 seconds. Discard the flow-through.
    d. Add 700 µL of Wash Buffer (diluted with ethanol) to the GeneJET™ purification column. Centrifuge for 30–60 seconds. Discard the flow-through and place the purification column back into the collection tube.
    e. Centrifuge the empty GeneJET™ purification column for an additional 1 minute to completely remove any residual wash buffer. *Note*: This step is essential as the presence of residual ethanol in the DNA sample may inhibit subsequent reactions.
    f. Transfer the GeneJET™ purification column to a clean 1.5 mL microcentrifuge tube (not included). Add 50 µL of Elution Buffer (10 mM Tris-HCl, pH 8.5) or nuclease-free water to the center of the GeneJET™ purification column membrane and centrifuge for 1 minute.
    g. Discard the GeneJET™ purification column and store the purified DNA at −20°C.
    h. Concentration can be measured by A260. If higher concentration is required a SpeedVac™ vacuum concentrator can be used.
    i. If needed, suspend DNA in TE buffer to desired concentration? (Introduction to Transfection | Bio-Rad)

TRANSFECTION

Electroporation instructions based on information from 90-0203 Gene Pulser Man (bio-rad.com), TrueTag Manual and 10009178b.qxp (bio-rad.com):

Grow cells:

Passage cells and start up a new flask with cells **one or two days** before electroporation. Grow to 70-90 % confluence before using for transfection.

IMPORTANT! Avoid creating bubbles while mixing and dispensing.

Day 1 – transfect cells

1. Prepare 6-well plate with media: Add 2000 µl of cell type-specific growth medium into two wells of the 6-well plate and place it in the 37°C incubator to pre-warm.

2. Prepare cells:
    a. If working with suspension cells, pellet the cells. If working with adherent cells, trypsinize the cells to detach them, add growth medium, and then pellet the cells.
    b. After pelleting the cells, remove the medium and wash the cells once with PBS without Ca^{2+} or Mg^{2+}, by carefully pipetting them. Take an aliquot and count the cells.
    c. Aliquot the number of cells needed to perform the experiment in Falcon tube. For a 500 µL reaction, use 500 000 adherent cells (1 x 10^6 cells/ml) and 1-1,5 million suspension cells (2-3 x 10^6 cells/ml).
    d. Pellet the cells by centrifugation at 100–400 × g for 5 minutes at room temperature. Start preparing step 3.

3. Prepare tube with TrueTag™ donor DNA + TrueCut™ Hifi Cas9 Protein + TrueGuide™ sgRNA in TE Buffer.
    a. Mix the TrueGuide™ sgRNA, TrueCut™ Hifi Cas9 Protein, TrueTag™ donor DNA and TE Buffer in a fresh, RNase-free microcentrifuge tube according to table 1 below. Mix well. *IMPORTANT! Maintain TrueCut™ Hifi Cas9 Protein:gRNA at a 1:1 molar ratio. Use high concentration TrueCut™ Hifi Cas9 Protein and ensure that the total volume of the RNP complex (TrueCut™ Hifi Cas9 Protein + gRNA) does not exceed 1/10th of the total reaction volume (e.g., 1 µL of Cas9 protein + gRNA in 10 µL total reaction volume).*
    b. Incubate the mix with TrueTag™ donor DNA + TrueCut™ Hifi Cas9 Protein + gRNA at room temperature for 5–20 minutes.

Table 1.

Reagent	Amount per cuvette
TrueCut™ Hifi Cas9 Protein	5 000 ng (37,5 pmol) = 1 µl (conc. 5 µg/µl)
TrueGuide™ sgRNA	960 ng (37,5 pmol) = 37,5 µl (conc. 1 µM)
Knockout Enrichment GFP-Puromycin	1 000 ng (1 pmol) = 6,9 µl (conc. 144,33 ng/µl)
Knockout Enrichment RFP-Blasticidin	1 000 ng (1 pmol) = 9,6 µl (conc. 103,63 ng/µl)

Amounts in table 1 based on information from TrueTag manual (x4), see below

Reagent	Amount per well of 24-well plate
TrueCut™ Cas9 Protein v2	1250 ng (7.5 pmol)
TrueGuide™ sgRNA	240 ng (7.5 pmol)
Knockout Enrichment GFP-Puromycin	250 ng (0.25 pmol)
Knockout Enrichment RFP-Blasticidin	250 ng (0.25 pmol)
Resuspension Buffer R	to 10 µL

4. While the mix with Cas9, gRNA and donor DNA incubates, continue with the tube from step 2 containing the cell pellet. Aspirate the PBS and resuspend the cells in 500 µL of Gene Pulser Electroporation Buffer (Bio-Rad). Gently pipette the cells to obtain a single cell suspension
5. Mix cells and Cas9+gRNA in electroporation cuvette. *IMPORTANT!* Avoid creating bubbles that can hinder electroporation.
    a. Pipette the cells in Electroporation Buffer (from step 4) up and down to resuspend any cells that might have settled at the bottom of the tube.
    b. Move the cell suspension to an electroporation cuvette.
    c. Add the Cas9+gRNA mix to the electroporation cuvette.
    d. Mix gently.

6. Electroporate using the Gene Pulser apparatus (Bio-Rad) with optimized conditions for the cell line.

7. After electroporation, transfer the contents of the cuvette into tissue culture dish with pre-warmed growth medium (from step 1).
    a. Incubate the cells in a humidified 37°C, 5% CO2 incubator for 48–72 hours. After 24 hr, change the growth medium.

Day 2-4 – verify editing

When the cells have reached 80-100% confluence, you are ready to proceed to the next steps. *Alternatively, if you wish to move to highly enriched populations directly, you can begin antibiotic selection.* The following assays can be done before and after selection to monitor the population. We recommend that a portion of the edited cells be maintained while the various tests are conducted. If more cells are needed for the tests, the cells can be split and grown up for a larger population.

Verify correct integration of tag by junction PCR and sequencing:

- We recommend confirmation of the correct insertion of the TrueTag™ cassette. This can be done by using junction primers on the pooled or clonal populations.
- On pooled populations, junction PCR (one primer on the genome and one in the cassette) only can tell you that the junction exists but is not accurate for the percentages. We recommend junction PCR over amplifying the full tag because PCR will be biased towards the smallest amplicon and will heavily enrich the short indel amplicons over the long tag amplicons.

Enrich with antibiotic selection:

- Once the cells have recovered and are reaching confluency, antibiotic selection can be used to further enrich the edited population. With antibiotic selection, a population of >99% positive cells can be obtained and used for downstream applications
    1. Split the cells into 5 aliquots for the following treatments.
        a Maintenance (no selection)
        b Antibiotic dose 1
        c Dose 2
        d Dose 3
    2. Maintain the no-selection cells as normal in case the selection fails.
    3. For the antibiotic selection we recommend using the optimal antibiotic killing dosage as determined by kill curve and also 1 dose above and 1 dose below the optimal killing dosage. Some endogenous promoters are weak and may produce only a low level of the antibiotic resistance gene so the lower dosage may still provide adequate selection.
    4. Maintaining the cells at these 3 doses for 5–10 days is usually sufficient for selection. Efficient killing of the unedited and recovery of the edited populations generally takes 5– 10 days. Enrichment can be monitored by microscope with GFP, RFP, BFP, or YFP tags.
    5. Once selection is completed, the cells can be assayed again to characterize the population.

Note that the effective range of blasticidin is broader than puromycin. A few dose combinations of puromycin and blasticidin are recommended for dual selection. After antibiotic selection for approximately 7 days, a significant population of cells should be both GFP- and RFP-positive.

Appendix 2: Genomic PCR protocol from the manufacturer

PB10.24

PCRBIOSYSTEMS

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PCRBIO Rapid Extract PCR Kit

Product description:

PCRBIO Rapid Extract PCR Kit combines rapid DNA extraction with fast, highly specific DNA amplification in a convenient, easy to use format. Eliminate the need for laborious and time-consuming DNA extraction methods with this simple, integrated extraction and amplification PCR kit powered by the latest advances in hot-start polymerase technology.

PCRBIO Rapid Extract PCR Kit has been developed for fast, efficient amplification of DNA from a variety of tissues and is particularly suited to solid tissue such as mouse tail or mouse ear. DNA extraction is performed in a single tube, removing the need for multiple washing steps. Extraction of DNA is rapid, providing DNA for PCR in 15 minutes. Extraction takes place in a single tube, minimizing potential contamination.

Extracted DNA is amplified using PCRBIO HS Taq Mix Red. Our antibody-mediated hot start polymerase uses the latest developments in polymerase technology and buffer chemistry to enhance PCR speed, yield and sensitivity. The completed reaction is ready for direct gel loading without the need to add loading buffer.

Component	80 reactions	400 reactions
5x PCRBIO Rapid Extract Buffer A	1 x 1.6 mL	5 x 1.6 mL
10x PCRBIO Rapid Extract Buffer B	1 x 800 µL	5 x 800 µL
2x PCRBIO HS Taq Mix Red	2 x 1.0 mL	10 x 1.0 mL

Shipping and storage

On arrival the kit should be stored between -30 °C and -15 °C. If stored correctly the kit will retain full activity for 12 months.

Limitations of product use

The product may be used for in vitro research purposes only.

Technical support

Help and support is available on our website at https://pcrbio.com/resources/ including answers to frequently asked technical questions. For technical support and troubleshooting you can submit a technical enquiry online, or alternatively email technical@pcrbio.com with the following information:

- Reaction setup
- Screen grabs of qPCR or PCR data.

Sample amounts

Sample	Amount per 100 µL extraction	Notes
Mouse tail clip	1 to 2 mm (2.5 to 6 mg)	
Mouse ear punch	2 to 4 mm^2 (2.5 to 6 mg)	
Animal tissue	3 to 30 mg	
Hair follicle	1-10 individual follicles	
Buccal swab	1 swab	Use 300 µL extraction volume for higher yield
Mammalian blood	2 to 8 µL Fresh/EDTA blood	2 mm^2 FTA, FTA elute or Guthrie cards
FFPE tissue	1 mm^3 or 2 mm^2 of 10 µm section	

Protocol

1. Extraction reaction setup

For each biological sample, create the following 100 µL extraction reaction:

Reagent	100 µL reaction	Notes
Mouse tail clip	1 to 2 mm (2.5 to 6 mg)	See table above for other samples
5x PCRBIO Rapid Extract Buffer A	20 µL	Lysis buffer
10x PCRBIO Rapid Extract Buffer B	10 µL	Protease containing buffer
PCR grade dH_2O	70 µL	

2. Extraction reaction incubation

Incubate extraction reaction for lysis, nuclease and protein denaturation, followed by heat-inactivation:

Cycles	Temperature	Time	Notes
1	75 °C	5 min	Vortex twice during incubation
1	95 °C	10 min	Deactivates protease

3. Dilute then centrifuge reaction

Add 900 µL PCR grade dH_2O to the deactivated reaction. Centrifuge at high speed in a microcentrifuge for 1 minute to pellet debris. Supernatant can be used directly in PCR or stored at -30 °C to -15 °C.

4. PCR Reaction setup

Prepare a master mix based on the following table:

Reagent	50 µL reaction	Final concentration
2x PCRBIO HS Taq Mix Red	25.0 µL	1x
Forward primer (10 µM)	2.0 µL	400 nM
Reverse primer (10 µM)	2.0 µL	400 nM
Supernatant from step 3	1.0 µL to 2.0 µL	variable
PCR grade dH_2O	Up to 50 µL final volume	

Cycle using conditions based on the following table:

Cycles	Temperature	Time	Notes
1	95 °C	1 min to 2 min	Initial denaturation and enzyme activation. For colony PCR increase denaturation time to 10 minutes
40	95 °C 55 °C to 65 °C 72 °C	15 seconds 15 seconds 1 to 90 seconds	Denaturation Anneal Extension (15 seconds per kb). For multiplex PCR use 90 seconds

Analyse by agarose gel electrophoresis. The 2x mix contains a red dye for tracking during agarose gel electrophoresis. In a 2% agarose TAE gel the dye migrates at a rate equivalent to 50-100 bp of DNA. In a 1% agarose TAE gel the dye migration rate is equivalent to 200-300 bp of DNA.

Version 2.6

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