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Original Article

Cloning Sheep Cytokin Genes and Preliminary Transfection to HT29 Cell Line

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Abstract: The consumption demand of people for terrestrial animal protein is increasing; therefore, to satisfy this, more effective pathogen-free animal production systems are required. Parasitic-infected ruminants have reduced the efficiency of food utilization. More seriously, the parasites could also lead to a chronic decrease in feed intake for the infected animals. The development of an effective anti-parasite vaccine requires knowledge of the host responses established by ruminants to expel GIN. The study was carried out to clone sheep cytokine genes for protein expression in mammalian cell culture. Once expressed, it is hypothesized that these proteins when added to a primary mast cell culture, will allow the growth of these cells. The IL3 and kit ligand genes were cloned into the bacterial expression pGEM–T easy vector to determine the correct sequences before being sub-cloned into the mammalian expression pWPI vector. The Pac 1 enzyme was used to cut restriction sites of the pGEM–T easy vector, the pWPI vector, IL3, and kit ligand genes in cloning strategies. The study also established 24 different conditions for transient transfection of IL3 and Kit ligand genes into HT29 cells and screened positive cells by flow cytometry. Results showed that clones of IL3 and kit ligand with the correct sequences were created using transient transfection. The transfection efficiency of IL3 and kit ligand DNA/pWPI plasmid into HT29 cells was extremely low, with approximately 9% for the best conditions. This low transfection efficiency leads to a shortage of IL3 and kit ligands for growing mast cells. Therefore, stable transfection will be required to have the highest efficiencies that produce the large quantities of IL3 and kit ligand proteins required for the culture of sheep mast cell lines.

Keywords: Sheep mast cells, growth factor, IL3, kit ligand, transient transfection, transfection efficiency.

1. Introduction

The world population has been gradually increasing, leading to higher food demands to satisfy the nutrition requirements for human beings. The demand for sustainable food production has seen renewed interest in satisfying the needs of an increasing world population. Proteins are a crucial food source in the diet of people, mainly originating from animals, along with a minority of plant

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resources. Animal protein products have a higher protein content compared to those from plants [1]. The demand for meat products has been projected to show a rapid increase by 2030, where a considerable proportion of meat products will come from livestock [2]. High-quality proteins are produced effectively and sustainably with minimally detrimental impacts on environments when suitably stocked and managed [3]. The demand of people for terrestrial animal protein is increasing; therefore, to satisfy this, more effective pathogen-free animal production systems are required. Parasitic infected ruminants have reduced the efficiency of food utilization [4]. More seriously, the parasites could also lead to a chronic decrease in feed intake for the infected animals [5]. Therefore, the presence of parasites closely relates to a reduction in productivity in livestock [6]. With sheep and goats in particular, it can be said that internal parasitism causes a reduction in livestock production efficiency.

The sheep industry in developed agricultural countries has been facing a principal health issue involving internal parasite infections [7, 8]. The efficiency of commercial anthelmintic treatment has been reduced because the resistance of internal parasites to anthelmintic treatment has increased, which led to a failure in control in some cases. The lower production and higher input costs have mainly been caused by decreasing anthelmintic treatment efficiency. This trend of growing global anthelmintic chemical resistance by internal parasites will influence the sustainable production of terrestrial animal protein for human consumption and therefore global food security. Therefore, single-dose vaccination may be considered as an effective method to supplement the anthelmintic control of gastrointestinal nematode (GIN) parasites. However, to develop the livestock industry sustainably, a balance between decreasing detrimental impacts on the environment and positive economic growth needs to be considered. Therefore, sustainable parasitic control should target less intensive, lower input, as well as reduce the risk of parasites causing economic losses associated with greater chances for combinations of all available control sources [9]. Finally, the introduction of an effective anti-parasite vaccine would satisfy the human demand for animal food products that are free of contaminating chemicals [10, 11]. In summary, there is a demand for an alternative measure to manage internal parasites more effectively, such as activation of the host immune system. However, the development of an effective anti-parasite vaccine requires knowledge of the host responses established by ruminants to expel GIN. This study was carried out to clone sheep cytokine genes and preliminary transfect to HT29 cell line serving for the development of mast cell culture in future research.

2. Material and Methods

2.1. Total RNA Isolation and cDNA Synthesis

Liquid nitrogen snap-frozen lung and thymus tissue from sheep was kindly provided by Dr Peter Hunt from the FD McMaster Laboratory, CSIRO, Armidale, NSW, and was obtained post-mortem from sheep under AEC number 12C8680. Total RNA was extracted from sheep's thymus and lung tissues using the RNeasy midi purification kit (Qiagen, USA) according to the manufacturer's instructions and then stored at -20 ºC until use. The purity and concentration of total RNA were determined spectrophotometrically, and the integrity of the RNA was determined by 1% agarose electrophoresis. Complementary DNA (cDNA) synthesis was performed using Superscript III First-Strand Synthesis SuperMix (Thermo Scientific, USA) according to the manufacturer's instructions. Briefly, 2 µg of total RNA was converted into cDNA using oligodT20 and random hexamer primers. The reaction contents were incubated at 25 ºC for 10 minutes, and then the tubes were incubated at 50 ºC for 30 minutes. After that, the reaction was terminated by heating at 85 ºC for 5 minutes and then chilled on ice. RNA was

removed from the cDNA by incubating the reaction tube with 2U of *E. coli* RNase H at 37 ºC for 20 minutes. The cDNA was stored at -20 ºC until use.

2.2. Amplification of IL3 and Kit Ligand from Ovine Tissues

Full-length IL3 and kit ligand were amplified from 200 ng of cDNA synthesized from RNA isolated from sheep thymus and lung tissues, respectively, using EconoTaq® Plus 2X Master Mix (Lucigen, USA) according to the manufacturer's instructions. Briefly, the reaction contents (EconoTaq Plus 2X Master Mix (25 µl), Forward primer (100 pmol/µl – 0.5 µl), Reverse Primer (100 pmol/µl – 0.5 µl), and DNA template (200 ng/µl) were added to a tube. The total volume was made up to 50 µL with free nuclease treated water. The cycle conditions for the PCR reaction were 94 ºC for 2 minutes, and then 94 ºC for 20 seconds, 62 ºC for 20 seconds, and 72 ºC for 1 minute, each repeated 34 times. The further extension at 72 ºC runs for 5 minutes; the final step is to hold templates at 4 ºC indefinitely. The primers used to amplify full-length IL3 were (Forward: ATGAGCAGCCTCTCTATCTT; Reverse: TCAGATAGTCTCTGCTGCT) and kit ligand (Forward: ACCATGAAGAAGACACAAACTTG; Reverse: TGGCTGCAACAGGG). The sizes of the IL3 and Kit Ligand PCR products were determined by TAE 1% agarose gel electrophoresis.

2.3. Cloning and Sequencing of Ovine IL3 and Kit Ligand

IL3 and Kit ligand PCR products were extracted from TAE 1% agarose gel using the EZ-10 Spin Column Gel Extraction kit (Bio Basic Inc., USA) following the manufacturer's instructions. The purity and concentration of the gel-extracted PCR products were determined by spectrophotometry at 260 and 280 nm using a Spectramax Me2 plate reader (Thermo Scientific, USA).

2.4. DNA Ligation

Ligation of PCR products into pGEM® –T Easy Vectors (Promega Company, USA) was performed according to the manufacturer's instructions. Briefly, 5µl of the 2× Rapid Ligation buffer (Promega, USA) was combined with pGEM®-T and the individual PCR products in a 1:3 molar ratio. T4 DNA ligase was added to the reaction tube to begin the ligation reaction, and the tubes were incubated overnight at 4 ºC in the PCR cycler.

2.5. Transformation of Competent XL1 Blue Cells

One liter of LB agar medium was prepared, sterilized by autoclave, and then put on a bench at room temperature to cool. After cooling, 500 µL Ampicillin (100 mg/ml) was well mixed with LB agar medium, and then the mixture was poured onto sterile plastic Petri dishes. Petri dishes were stored at 4 ºC until required. Chemically competent XL1 blue cells (Stratagene, USA) were transformed with 2 µl of the ligation reaction products of IL3/pGEM®-T easy or Kit Ligand/pGEM®-T easy. Briefly, 50 µl of competent XL1 blue cells were thawed on ice for 5 minutes, incubated with the ligation reaction products, and incubated on ice for a further 30 minutes in 15 ml falcon tubes. The cells were shock-heated for 30 seconds at exactly 42 ºC in the water bath and immediately returned to ice for 2 minutes. SOC medium (950 µL) was added to each transformation reaction, which was incubated for 1 hour in a shaking incubator at 220 rpm at 37 ºC. 100 µL of cell suspension was spread on LB ampicillin IPTG/XGal agar plates, and the plates were incubated overnight at 37 ºC. Blue white colony screening in combination with colony PCR assays and agar patch plates was used to identify IL3 and kit ligand positive XL1 Blue colonies. Eighteen white colonies from each of the IL3 or kit ligand transformation plates were transferred onto an Ampicillin LB agar containing a printed square grid, and the remainder of each colony was used as the template in the colony PCR assay. M13 forward

(CAGGAAACAGCTATGAC) and reverse (CCCAGTCACGACGTTGTAAAACG) primers were used in the colony PCR assay. The cycle conditions for the colony PCR were 94 ºC for 2 minutes, followed by 34 cycles of 50 °C for 20 seconds, 72 ºC for 2 minutes, and then further extension at 72 ºC for 5 minutes. The PCR products were resolved on a TAE 1% agarose gel. The PCR products were purified using an EZ-10 PCR clean-up kit (Bio Basic Inc., USA) and sent to the Australian Genome Research Facility (AGRF) for sequencing using M13 forward or reverse primers.

Colonies that contained the correct size PCR products were expanded from the patch plate in 10 ml of liquid LB ampicillin media and grown overnight at 37 ºC at 220 rpm in a shaking incubator. From this culture, glycerol stocks were made and archived at -80 ºC to preserve the plasmid. The remainder of the overnight cultures were centrifuged for 10 minutes (3,000 g) to collect bacterial cell pellets in preparation for plasmid purification. Plasmids were isolated from these colonies using the EZ-10 plasmid purification kit (Bio Basic Inc., USA) according to the manufacturer's instructions. Briefly, pellets were resuspended in 100 µL of cell suspension buffer and mixed well until homogenate solutions were kept for 1 minute at room temperature. The mixture was transferred to 1.5 ml centrifuge tubes and then added with 200 µL of solution II and incubated at room temperature for 1 minute. 350 µL of Solution III was added and incubated at room temperature for 1 minute. The mixture was then centrifuged for 5 minutes (12,000 rpm), and transferred supernatant to EZ-10 columns to centrifuge for 2 minutes (12,000 rpm). The flow through was discarded, and 750 µL of Wash solution was added to clean DNA plasmid using centrifugation at 2 minutes (10,000 rpm). The columns were then transferred to clean 1.5 ml collection tubes, and 50 µL of elution buffer was added to the column and incubated at room temperature for 2 minutes before centrifuging at 10,000 rpm for 2 minutes. The purified plasmids were stored at -20 ºC until required.

2.6. Subcloning of Kit Ligand 1 and IL3-2 into pWPIvector

The purified DNA expression plasmid pWPI, pGEM®T-IL3, and pGEM®-T Kit ligand plasmids were cut overnight with restriction enzyme Pac1 (NEB, USA) at 37 ºC to create the pWPI Pac1 insertion site and to release IL3 and Kit ligand from the pGEM®-T plasmid. The cut plasmid, IL3, and kit ligand inserts were purified using TAE 1% agarose gel electrophoresis and isolated from the gel via the EZ-10 Spin Column Gel Extraction kit (Bio Basic Inc., USA) following the manufacturers' instructions. The purified DNAs of IL3 and Kit Ligand were subcloned into pWPI lentiviral expression vectors using an instant sticky end ligase master mix (NEB, USA). 1 µl of the pWPI/IL3 or pWPI/kit ligand ligation reactions were transformed into XL-1 blue as described above.

2.7. Cloning Strategy

The strategy chosen to clone IL3 and kit ligand into the pWPI mammalian expression is summarized in Figure 3. The cloning strategy involved amplifying IL3 and kit ligand genes from ovine thymus and lung mRNA (cDNA), respectively, and attaching Pac1 restriction sites onto the 5' and 3' ends of the genes during the amplification process (Figure 1). TA cloning was used to insert these genes into the pGEM®-Teasy plasmid for M13 Sanger sequence analysis. Once a clone with the correct sequence was identified, Pac1 was used to digest IL3-pGEM®-T and kit ligand-pGEM thereby releasing the IL3 and kit ligand genes out of pGEM®-T plasmids. These genes were ligated into the Pac1 digested pWPI mammalian expression vector in preparation for expression in a mammalian cell line.

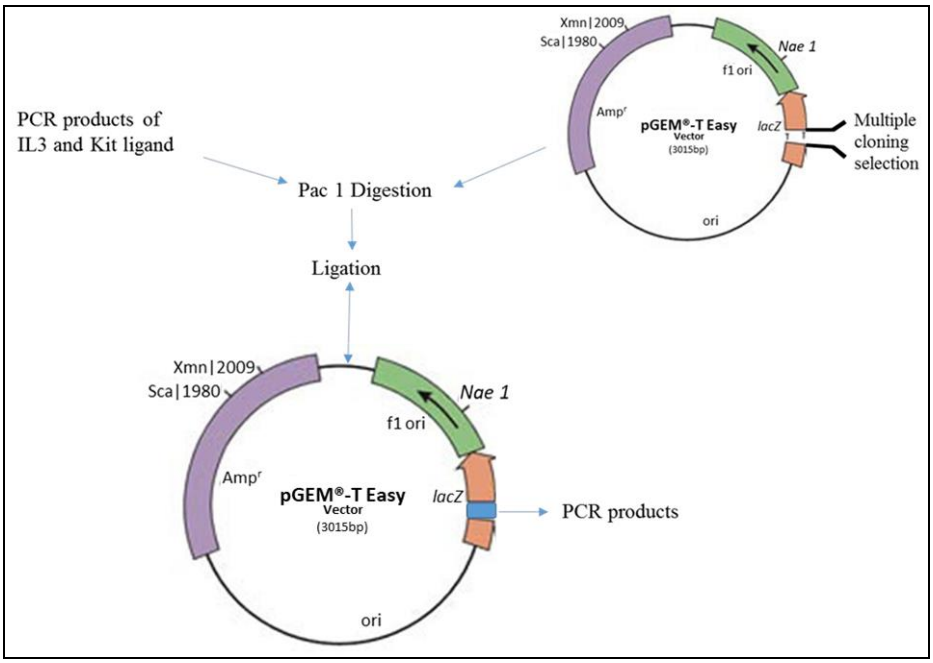

Figure 1. Cloning strategy of PCR products of IL3 and Kit ligand genes into pGEM®- T plasmid, purified PCR products, and pGEM®- T were digested with Pac 1 enzyme to allow cloning genes inserted into the plasmid. Digestive products were ligated and then transformed into XL blue 1 competent cell.

2.8. Midi-Scale Plasmid Purification of pWPI-IL3 and pWPI-Kit Ligand

Single white colonies were chosen and transferred to 10 ml LB media consisting of 10 µl (with a concentration of 50µg/ml ampicillin. The bacterial colony was cultured in a shaking incubator (220 rpm) at 37 ºC for approximately 18 hours. From this culture, glycerol stocks were made and archived at -80 ºC to preserve the pWPI plasmid, consisting of IL3 and kit ligand DNA, until required.

The rest of the culture media was then transferred to a larger quantity with 100 ml of LB media, containing the same antibiotics, and incubated at 37 ºC for 18 hours in the shaking incubator (220 rpm). Bacterial cells were collected by centrifuging the overnight LB culture at 12,000 g for 3 minutes for preparation of plasmid purification. DNA plasmid was isolated using the Jetstar 2.0 plasmid midi kit (Gentaur, USA) following the manufacturer's instructions. Briefly, 8 ml cell resuspending buffer (E1) was added to the pellets to resuspend and mixed well until a homogenous solution was achieved The mixture was added with 8 mL lysis buffer (E2) to lyse bacterial cells until homogeneous mixture and kept for 5 minutes at room temperature. 8 mL precipitation buffer (E3) was added to the mixture and inverted until homogeneous mixture. The precipitation mixture was transferred to an equilibrated column to remove the solution. 20 ml of wash solution (E5) was added to the equilibrated column to clean the DNA plasmid and flow-through was discarded. 5 ml of elution buffer (E6) was added to the column to elute DNA plasmid which was collected into a sterile 15 ml centrifuge. 3.5 ml of isopropanol was then mixed to precipitate plasmid. The mixture was centrifuged at 12,000 g for 30 minutes at 4 ºC to precipitate plasmid. DNA pellets were then washed with 3 ml of 70% ethanol, and centrifuged tubes at 12,000 g for 5 minutes at 4 ºC. Pellets were re-suspended in 50 µl of TE buffer. Plasmid concentration was identified by spectrometry with absorbance at 260 nm, and purified DNA plasmid was stored until require.

2.9. Culture and Electroporation-Based Transfection of HT29 Cells

The HT29 colorectal cancer cell line was grown in DMEM (Thermo, USA) supplemented with 10% FCS (Bovogen, Australia) and 1x P/S (Thermo, USA). The cells were routinely passaged with 0.25% Trypsin-EDTA (Bovogen, Australia) and incubated at 37 ^{o}C in a humidified 95% air: 5% CO_2 atmosphere. To introduce the Kit Ligand-pWPI plasmid into the HT29 cells, the cells were electroporated using a Neon electroporation system (Thermo, USA) according to the manufacturer's instructions. Briefly, the HT29 cells were trypsinized from the culture flask, washed twice with phosphate buffered saline (PBS), and resuspended at $1x10^5$ cells in 10 µl of buffer R containing 1 µg of Kit Ligand-pWPI plasmid and incubated for less than 30 minutes at room temperature. To determine the optimum electroporation conditions with 93% for primary fibroblasts, a 24-well screen was performed. After the cells were electroporated, they were immediately transferred into prewarmed, complete media without antibiotics and incubated as described above. Six hours after electroporation, antibiotics were added to the cultured cells, and the cells were incubated at 37 ^{o}C in a humidified 95% air: 5% CO_2 atmosphere until required for flow cytometric analysis.

2.10. Flow Cytometric Analysis

Four days after electroporation of HT29 cells, the Kit Ligand-pWPI transfection efficiencies were determined using flow cytometry. HT29 cells were detached from the wells of the 24 well culture plate using 0.25% trypsin-EDTA and resuspended in ice-cold Hank's balanced salt solution (Thermo, USA). Immediately before flow cytometric analysis, the cells were incubated with 1 µg/ml propidium iodide to determine the viability status of the cells. Transfected HT29 cells (5,000 events) were acquired on an AmnisFlowSight flow cytometer (Millipore, USA) and the data was analysed using IDEAS software version 6.0.340.0 (Amnis Corporation, USA). The percentage of cells expressing GFP was determined using a FACScan flow cytometer (Becton Dickinson Biosciences). This assay is approximately 48% more sensitive than the fluorescence microscope assay.

3. Results and Discussion

3.1. Bioinformatic Predicted Tissue Expression of IL3 and Kit Ligand

In an attempt to identify the ovine organs or tissues that were predicted to express significant IL3 and kit ligand mRNA transcripts, the Unigene database was searched (Tables 1 and 2 respectively).

Table 1. The predicted expression of IL3 transcripts in tissues and organs from different species

Species	Kit Ligand Unigene Accession Number	Tissue	Transcripts per Million (TPM)	Gene EST/Total EST in Pool
Mouse	256118 – Mm.983	Embryonic Thymus	1	1/677554
			8	1/121153
Human	130809 – Hs.694	No entry		
Bovine	30440 – Bt.538	No entry		
Ovine	1299686 – Oar.437	No entry		

Based on the different species database in Table 1, it is well documented that thymus tissues are the best organs to amplify IL3 due to the significantly larger number of transcripts per million (TPM) and gene EST per total EST in the pool as well. Similarly, lung tissues were used to amplify kit ligands because of the relatively larger number of transcripts per million in comparison with other tissues such as liver, thymus, spleen, intestine and skin (Table 2).

Table 2. The predicted expression of kit ligand transcripts in tissues and organs from different species

Species	Kit Ligand Unigene Accession Number	Tissue	Transcripts per Million (TPM)	Gene EST/Total EST in Pool
Mouse	271246 – Mm.45124	Lung	400	40/99799
		Liver	80	9/111370
		Thymus	66	8/121153
		Spleen	43	4/92417
Rat	401268 – Rn.44216	Lung	175	4/22829
		Liver	0	0
		Thymus	0	0
		Spleen	0	0
Bovine	30311 – Bt.354	Lung	85	2/23399
		Intestine	14	1/68828
		Skin	29	1/33886
Ovine	No entry			

3.2. Isolation of Total RNA from Sheep

Based on the comparative predicted expression of the IL3 and kit ligand transcripts, total RNA was isolated from the thymus and the lung of sheep (Figure 2A). A greater quantity of RNA was isolated from the lung than the thymus, even though approximately the same tissue wet weight was used. The 28S and 18S ribosomal RNA were present in the total RNA isolated from the thymus and lung tissues of sheep. The 28S ribosomal RNA band was present at approximately twice the amount of the 18S ribosomal band and indicated that the total RNA extracted from these tissues was not degraded. Also, the 260:280nm absorbance ratio of each of the total RNA preparations from the tissues was between 1.9 and 2.1, suggesting that the total RNA isolated from these tissues was sufficiently pure. Therefore, the quality of the total RNA preparation was high enough to produce good quality cDNA (Figure 2B) for full-length IL3 and Kit ligand gene amplification.

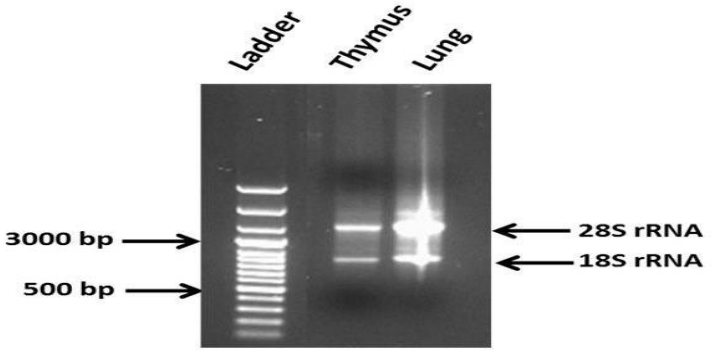

Figure 2. 1% TAE agarose gel of total RNA extracted from the thymus and lung tissues of the sheep.

3.3. Amplification of Ovine IL3 and Kit Ligand

The PCR amplification products from the cDNA templates for the thymus and the lung amplified multiple bands when IL3 and kit ligand primers were used (Figure 3). The predicted sizes of the IL3 and kit ligand PCR products were 441 bp and 577 bp, respectively. Bands corresponding to the predicted sizes for IL3 and kit ligand were amplified from thymus and lung tissues, respectively. Therefore, these bands were cut out purified and used in preparation for TA cloning into the pGEM® -T easy plasmids.

The ligation reaction products were transformed into XL 1 blue and spread on petri dishes to collect white colonies. White colonies were selected to run PCR assays to check positivity. Eighteen white colonies were collected from transformation reactions that were used to amplify PCR reactions. The colonies with number 1 and 8 for IL3 and number 9 for Kit Ligand were not chosen due to differences in sizes when compared to 1kb ladders and other colonies.

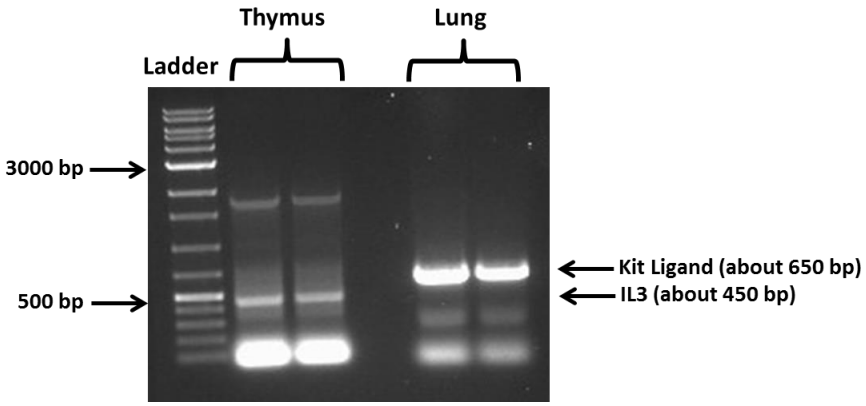

Figure 3. Amplification of IL3 and Kit ligand from ovine thymus and lung cDNA, respectively. A 1% agarose gel showed the amplification products when primers designed to amplify full-length IL3 and kit ligand from cDNA of sheep thymus and lung tissues were used. Bands corresponding to the predicted sizes of IL3 and kit ligand full-length genes were present.

The images of 1% agarose gel from the two Figures 4A and 4B indicated that negative colonies were found in colony 1 and 8 for IL3 gene and colony 9 for kit ligand gene. These correct colonies chosen were then sequenced by Sanger sequence. These sequences were then compared with all species using Basic Local Alignment Search Tool (BLAST) method to identify the right sequences of both IL3 and Kit Ligand colonies. Results from the blast of Kit Ligand and IL3 colonies are shown in Table 3.

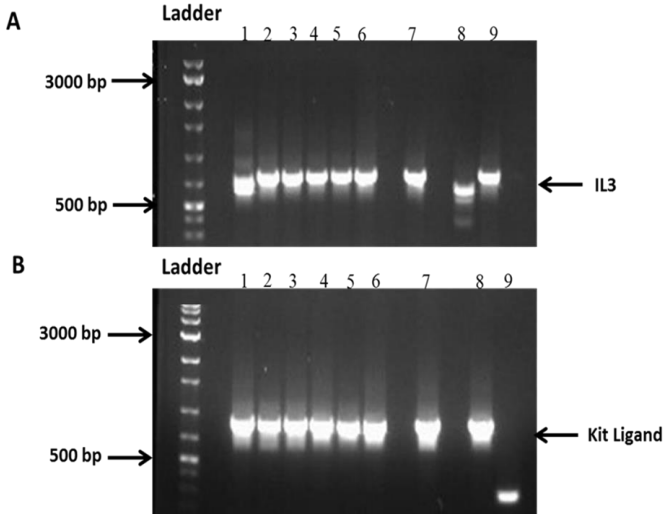

Figure 4. PCR colony products of IL3 and Kit Ligand in pGEM® - T vector.

Table 3. Blast identity matches of colonies qRT– PCR products amplified from cDNA

<table>
<tr><th>Clone</th><th>Blast Alignment</th><th>Identity Similarity</th></tr>
<tr><td>Kit 1</td><td>Query 131
ATGAAGAAGACACAAACTTGGATTATCACTTGCATTTATCTTCAACTGCTCCTAT
TTAAT 190
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 16
ATGAAGAAGACACAAACTTGGATTATCACTTGCATTTATCTTCAACTGCTCCTAT
TTAAT 75

Query 671 ACAAAACCATTTATGTTACCCCCTGTTGCAGCCA 704
||||||||||||||||||||||||||||||||||
Sbjct 556 ACAAAACCATTTATGTTACCCCCTGTTGCAGCCA 589</td><td>569/574
(99%)
Plus/Plus</td></tr>
<tr><td>Kit 2</td><td>Query 118
ATGAAGAAGACACAAACTTGGATTATCACTTGCATTTATCTTCAACTGCTCCTAT
TTAAT 177
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 16
ATGAAGAAGACACAAACTTGGATTATCACTTGCATTTATCTTCAACTGCTCCTAT
TTAAT 75
Query 658 ACAAAACCATTTATGTTACCCCCTGTTGCAGCCA 691
||||||||||||||||||||||||||||||||||
Sbjct 556 ACAAAACCATTTATGTTACCCCCTGTTGCAGCCA 589</td><td>565/574
(98%)
Plus/Plus</td></tr>
<tr><td>Kit 3</td><td>Query 124
ATGAAGAAGACACAAACTTGGATTATCACTTGCATTTATCTTCAACTGCTCCTAT
TTAAT 183
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 16
ATGAAGAAGACACAAACTTGGATTATCACTTGCATTTATCTTCAACTGCTCCTAT
TTAAT 75
Query 664 ACAAAACCATTTATGTTACCCCCTGTTGCAGCCA 697
||||||||||||||||||||||||||||||||||
Sbjct 556 ACAAAACCATTTATGTTACCCCCTGTTGCAGCCA 589</td><td>570/574
(99%)
Plus/Plus</td></tr>
<tr><td>Kit 4</td><td>Query 121
TGGCTGCAACAGGGGGTAACATAAATGGTTTTGTGACACTGACTCTGGAATCTTT
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Sbjct 589
TGGCTGCAACAGGGGGTAACATAAATGGTTTTGTGACACTGACTCTGGAATCTTT
TTCAG 530
Query 661 TGCAAGTGATAATCCAAGTTTGTGTCTTCTTCAT 694
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(99%)
Plus/Minus</td></tr>
<tr><td>Kit 5</td><td>Query 118
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Sbjct 589
TGGCTGCAACAGGGGGTAACATAAATGGTTTTGTGACACTGACTCTGGAATCTTT
TTCAG 530</td><td>570/574
(99%)
Plus/Minus</td></tr>
</table>

<table>
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(99%)
Plus/Minus</td></tr>
<tr><td>Kit 7</td><td>Query 118
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Sbjct 16
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TTAAT 75
Query 658 ACAAAACCATTTATGTTACCCCCTGTTGCAGCCA 691
||||||||||||||||||||||||||||||||||
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(99%)
Plus/Plus</td></tr>
<tr><td>Kit 8</td><td>Query 118
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TTAAT 177
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Sbjct 16
ATGAAGAAGACACAAACTTGGATTATCACTTGCATTTATCTTCAACTGCTCCTAT
TTAAT 75
Query 658 ACAAAACCATTTATGTTACCCCCTGTTGCAGCCA 691
||||||||||||||||||||||||||||||||||
Sbjct 556 ACAAAACCATTTATGTTACCCCCTGTTGCAGCCA 589</td><td>570/574
(99%)
Plus/Plus</td></tr>
<tr><td>IL3-2</td><td>Query 131
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CTCA 190
||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct 43
CATGAGCAGCCTCTCTATCTTGCATCTGCTCCTGCTCCTGCTTTCTCTCCATGCCC
CTCA 102
Query 551 AGCAGCAGCAGAGACTATCTGA 572
||||||||||||||||||||||
Sbjct 463 AGCAGCAGCAGAGACTATCTGA 484</td><td>441/442
(99%)
Plus/Plus</td></tr>
<tr><td>IL3-3</td><td>Query 117
TCAGATAGTCTCTGCTGCTGCTAAGTCGTGTCTGAGATAGCCTCTAATGGTAGCC
AAATA 176
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 484
TCAGATAGTCTCTGCTGCTGCTAAGTCGTGTCTGAGATAGCCTCTAATGGTAGCC
AAATA 425 Query 537 CAAGATAGAGAGGCTGCTCATG 558
||||||||||||||||||||||
Sbjct 64 CAAGATAGAGAGGCTGCTCATG 43</td><td>440/442
(99%)
Plus/Minus</td></tr>
</table>

<table>
<tr><td>IL3-4</td><td>Query 120
TCAGATAGTCTCTGCTGCTGCTAAGTCGTGTCTGAGATAGCCTCTAATGGTAGCC
AAATA 179
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 484
TCAGATAGTCTCTGCTGCTGCTAAGTCGTGTCTGAGATAGCCTCTAATGGTAGCC
AAATA 425 Query 540 CAAGATAGAGAGGCTGCTCATG 561
||||||||||||||||||||||
Sbjct 64 CAAGATAGAGAGGCTGCTCATG 43</td><td>442/442
(100%)
Plus/Minus</td></tr>
<tr><td>IL3-5</td><td>Query 126
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AAATA 185
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 484
TCAGATAGTCTCTGCTGCTGCTAAGTCGTGTCTGAGATAGCCTCTAATGGTAGCC
AAATA 425
Query 546 CAAGATAGAGAGGCTGCTCATG 567
||||||||||||||||||||||
Sbjct 64 CAAGATAGAGAGGCTGCTCATG 43</td><td>441/442
(99%)
Plus/Minus</td></tr>
<tr><td>IL3-6</td><td>Query 118
CATGAGCAGCCTCTCTATCTTGCATCTGCTCCTGCTCCTGCTTTCTCTCCATGCCC
CTCA 177
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 43
CATGAGCAGCCTCTCTATCTTGCATCTGCTCCTGCTCCTGCTTTCTCTCCATGCCC
CTCA 102
Query 538 AGCAGCAGCAGAGACTATCTGA 559
||||||||||||||||||||||
Sbjct 463 AGCAGCAGCAGAGACTATCTGA 484</td><td>441/442
(99%)
Plus/Plus</td></tr>
<tr><td>IL3-7</td><td>Query 131
CATGAGCAGCCTCTCTATCTTGCATCTGCTCCTGCTCCTGCTTGCTCTCCATGCCC
CTCA 190
||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct 43
CATGAGCAGCCTCTCTATCTTGCATCTGCTCCTGCTCCTGCTTTCTCTCCATGCCC
CTCA 102
Query 551 AGCAGCAGCAGAGACTATCTGA 572
||||||||||||||||||||||
Sbjct 463 AGCAGCAGCAGAGACTATCTGA 484</td><td>441/442
(99%)
Plus/Plus</td></tr>
<tr><td>IL3-9</td><td>Query 124
CATGAGCAGCCTCTCTATCTTGCATCTGCTCCTGCTCCTGCTTTCTCCCCATGCCC
CTCA 183
||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct 43
CATGAGCAGCCTCTCTATCTTGCATCTGCTCCTGCTCCTGCTTTCTCTCCATGCCC
CTCA 102
Query 544 AGCAGCAGCAGAGACTATCTGA 565
||||||||||||||||||||||
Sbjct 463 AGCAGCAGCAGAGACTATCTGA 484</td><td>441/442
(99%)
Plus/Plus</td></tr>
</table>

The positive colonies with the highest identities were selected for both IL3 and Kit Ligand. The Kit Ligand for colony 1 with the highest identity of 99% and positivity meanwhile, the selective colony for IL3 was number 2 with the highest identity and positivity, were selected and then used for ligation reactions into pWPI lentivirus mammalian expression vector. The DNA recombinant plasmids of pGEM® -T vectors for IL3 and Kit Ligand and pWPI plasmid were cut with Pac1 before ligating into the pWPI lentivirus expression vector. The cut DNA fragments of IL3 -2 and Kit Ligand 1 were determined and extracted from a 1% agarose gel with correct gel bands.

The DNA fragments extracted from the gel slice are shown in Figure 5. The extracted products of DNA fragments were ligated into pWPI mammalian expression vector to convert DNA of IL3 and kit ligand into proteins by the transfection method.

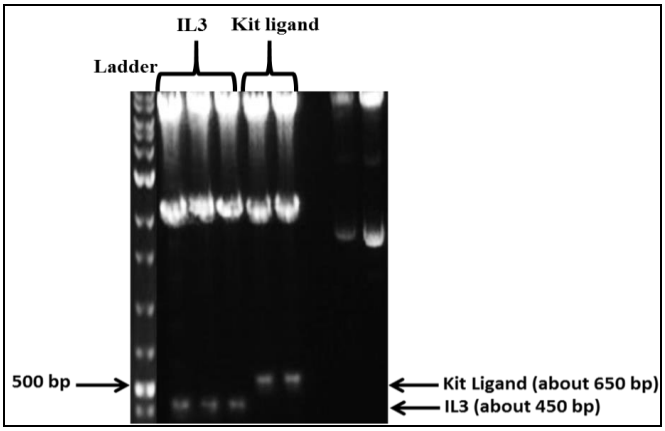

Figure 5. DNA fragments of IL3 and Kit Ligand cut with Pac1 enzyme observed on a 1% agarose gel.

3.4. Transfection Efficiency of Kit Ligand and IL3 Ligating into pWPI Vector

Figure 6 illustrates some pictures of the cells with and without fluorescence that also proves the positivity and negativity of cells. Ch01 bright field has cells in it without fluorescence; nonetheless, Ch02 (the GFP channel) has the same cells as Ch01 but has green fluorescence in it. This demonstrates that Ch01 is GFP positive, which expresses the Kit ligand. Similarly, Ch03–the PI channel, also has the same cell in it; however, there is no fluorescence.

Therefore, the same GFP cells for Ch01 and Ch02 were positive, and the PI cell for Ch03 with no fluorescence was negative. The cells with PI negative and GFP positive are alive and viable transfected cells.

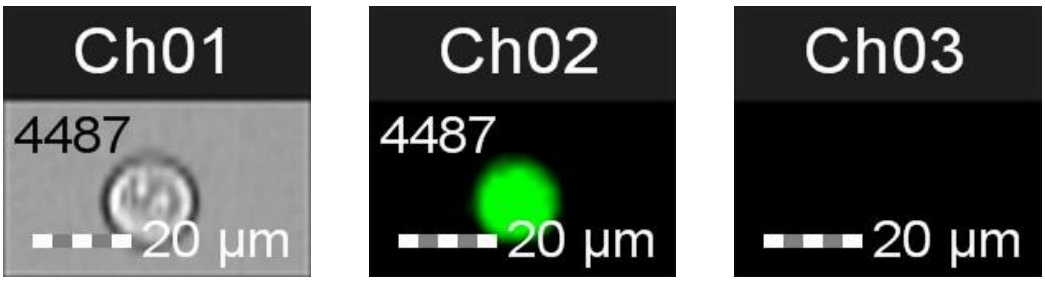

Figure 6. The intact cells with fluorescence expressed the positivity and negativity.

Figure 7 illustrates the transfection efficiencies of the 24-well screen. The greatest transfection efficiency of approximately 9% was produced by a single pulse with a voltage of 1100V and a 30 second pulse width in well number 6. Meanwhile, transfection efficiencies were observed from the different well conditions, such as wells 1, 4, 5, 9, 14, and 23 were equal to zero due to these conditions killing the cells. However, the 9% highest transfection efficiency collected was still too low to use transient transfection of HT29 to produce enough Kit ligand protein for the mast cell culture. Therefore, to produce Kit Ligand and IL3 proteins for the mast cell culture, a stably transfected HT29 clone that expresses the recombinant proteins using the transfection conditions defined above may be required. This will help us to have more Kit Ligand and IL3 that are used for the mast cell culture.

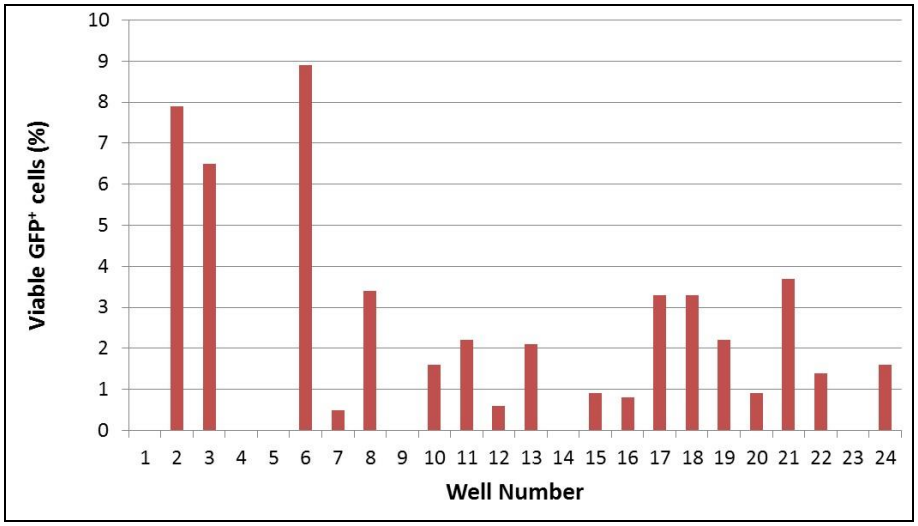

Figure 7. Transfection efficiencies of 24 different conditions.

4. Discussion

In this study, we used the pWPI vector, which is a large 11,000 bp mammalian lentiviral transfer plasmid, to clone IL3 and Kit ligand genes for two main reasons. First of all, this vector consists of the EF-1 alpha promoter, which allows us to achieve a robust, constitutive expression of IL3 and kit ligand genes and switch on IL3 and kit ligand genes expressed in the single mRNA. This vector also contains an internal ribosome entry site (IRES), which is a sequence in the mRNA that allows for translation initiation by the ribosome in the middle of mRNA transcripts during protein synthesis (translation). Thus, the IRES site was used to make two separate proteins (e.g. IL3 and GFP) from a single IL3-GFP mRNA molecule. The IL3 and kit ligand protein products are difficult to be detected in a mixture, whereas we can quantify the concentration of GFP proteins by using flow cytometry to select cells. The appearance of IRES is extremely important because we need to separate IL3 and kit ligand proteins out of a mixture that will be used to grow mast cells. Secondly, the pWPI vector also comprises LTR sites that allows integration of EF–1 alpha promoter, IL3 and kit ligand genes, and the GFP gene into the genome of mammalian cells such as HT29 cells. This will give us the option of creating stable transfection instead of transient transfection in case transient transfection does not work well.

The transient transfection efficiencies were carried out in the twenty-four well conditions to find out the optimal conditions for transfection of Kit ligand and IL3 into HT29 cells. The transfection efficiencies in this experiment were

extremely low compared with the Neon transfection system database. According to the instructions of the Neon transfection system for HT-29 cells, the high transfection efficiency reached 71% for conditions compatible with a pulse voltage of 1,300V, a pulse width of 20 ms, and a pulse number of 2 in tip type 10 µl. Meanwhile, 82% of transfection efficiency was reached at the conditions with 1,650 pulse voltage, pulse width 10, and 3 pulse number. The results of the database on transfection efficiency were gained after 24 hours of transfection, whereas transfection efficiencies of IL3 and Kit ligand were obtained after 72 hours of transfection. In a normal mechanism, the longer time for transfection will lead to higher transfection efficiency because the cell density is higher, which helps increase transfection efficiencies. In addition, the culture condition in our experiment was the same as the instructions of the Neon transfection system, with a temperature of 37 ºC, atmosphere air of 95%, and carbon dioxide (CO_2) of 5%. An important reason directly affecting the transfection efficiency of HT-29 is the size of the plasmid used, which was not mentioned in the Neon transfection system. In this study, 24 different well conditions were conducted to optimize the transfection efficiency; however, most of them obtained extremely low efficiencies under 4% viable cells. All of the wells used the same pWPI plasmid, which has a large size of approximately 11,000 bp in different conditions. Therefore, it is likely that the size of the plasmid has the strongest influence on transfection efficiency because the bigger size of the plasmid will make it more difficult to transfect the Kit ligand and IL3 into HT-29 cells. This is considered a major cause of very low transient transfection efficiency in this experiment. For the above reasons, stable transfection may require large plasmids like pWPI for cell expression instead of transient transfection in the viral environment. The successful transfection of this plasmid pWPI containing IL3 and Kit ligand to collect proteins is necessary because they are important growth factors for culturing mast cells. The transfection efficiency of this study was relatively lower than that reported by [12] when transfection of mammalian cells using linear polyethylenimine. As explained before, our results can be improved further when smaller size of plasmid is used for cloning. The experiments performed were the successful amplification and cloning of two sheep cytokines into a mammalian expression vector (pWPI) and to find out which conditions are the most suitable for transient transfection. So, the research has produced two important reagents that are required to successfully culture mast cells in vitro. Further research will be needed with stable transfection to get a huge amount of IL3 and Kit ligand proteins for the culture of mast cells.

5. Conclusion

Sheep IL3 and Kit ligand genes were successfully amplified from thymus and lung tissue, respectively, cloned into pGEM plasmid and sequenced. The genes were subcloned into the mammalian pWPI expression vector. The large size of the plasmid strongly affected the low level of transfection efficiency. Therefore, transient transfection was not good enough to produce the quantity of recombinant proteins needed for mast cell culture. That is why stable transfection is required. This involves transfecting the cells using conditions that were produced by a single pulse with a voltage of 1100 V and a 30-second pulse width in well number 6, bringing out the highest transfection efficiency with approximately 9% of viable GFP+ cells and cloning the cells out to identify cells that stably expressed plasmid. It is estimated that a stable clone will take three to six months to be produced.

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