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# A novel universal probe library quantitative reverse transcription polymerase chain reaction method to profile immunological gene expression in blood of captive beluga whales ( *Delphinapterus leucas* )

Ming-An Tsai <sup>1</sup> , I-Hua Chen <sup>2</sup> , Jiann-Hsiung Wang <sup>2</sup> , Shih-Jen Chou <sup>2</sup> , Tsung-Hsien Li <sup>1</sup> , Ming-Yih Leu <sup>1</sup> , Hsiao-Kuan Ho <sup>3</sup> , Wei Cheng Yang <sup>Corresp. 2</sup>

<sup>1</sup> Department of Biology, National Museum of Marine Biology and Aquarium, Pingtung, Taiwan

<sup>2</sup> College of Veterinary Medicine, National Chiayi University, Chiayi, Taiwan

<sup>3</sup> Department of Biology, Hi-Scene World Enterprise Co Ltd., Pingtung, Taiwan

Corresponding Author: Wei Cheng Yang  
Email address: jackywc@gmail.com

Cytokines are fundamental for a functioning immune system, and thus, potentially serve as important indicators of animal health. Quantitation of mRNA using quantitative reverse transcription polymerase chain reaction (qRT-PCR) is an established immunological technique. It is particularly suitable for detecting the expression of proteins against which monoclonal antibodies are not available. In this study, we developed a novel probe-based quantitative gene expression assay for immunological assessment of captive beluga whales ( *Delphinapterus leucas* ) that is one of the most common cetacean species on display in aquariums worldwide. Six immunologically relevant genes (IL-2R $\alpha$ , -4, -10, -12, TNF $\alpha$ , and IFN $\gamma$ ) were selected for analysis, and two validated housekeeping genes (PGK1 and RPL4) with stable expression were used as reference genes. Sixteen blood samples were obtained from four animals with different health conditions and stored in RNAlater solution. These samples were used for RNA extraction followed by qRT-PCR analysis. Analysis of gene transcripts was performed by relative quantitation using the comparative C<sub>q</sub> method with the integration of amplification efficiency and two reference genes. The expression levels of each gene in the samples from clinically healthy animals were normally distributed. Transcript outliers for IL-2R $\alpha$ , IL-4, IL-12, TNF $\alpha$ , and IFN $\gamma$  were noticed in four samples collected from two clinically unhealthy animals. This assay has the potential to identify immune system deviation from normal state, which is caused by health problems. Furthermore, knowing the immune status of captive cetaceans could help both trainers and veterinarians in implementing preventive approaches prior to disease onset.

1 **A novel universal probe library quantitative reverse transcription polymerase**  
2 **chain reaction method to profile immunological gene expression in blood of**  
3 **captive beluga whales (*Delphinapterus leucas*)**

4

5 Ming-An Tsai<sup>1</sup>, I-Hua Chen<sup>2</sup>, Jiann-Hsiung Wang<sup>2</sup>, Shih-Jen Chou<sup>2</sup>, Tsung-Hsien Li<sup>1</sup>, Ming-Yih

6 Leu<sup>1</sup>, Hsiao-Kuan Ho<sup>3</sup>, Wei-Cheng Yang<sup>2</sup>

7 <sup>1</sup>National Museum of Marine Biology and Aquarium, 2 Houwan Road, Checheng, Pingtung, 944,  
8 Taiwan.

9 <sup>2</sup>Department of Veterinary Medicine, National Chiayi University, 580 Xinmin Road, Chiayi, 600,  
10 Taiwan

11 <sup>3</sup>Hi-Scene World Enterprise Co Ltd., 2 Houwan Road, Checheng, Pingtung, 944, Taiwan.

12 Corresponding author:

13 Wei-Cheng Yang<sup>2</sup>

14 Email address: [jackywc@gmail.com](mailto:jackywc@gmail.com)

## 16 Abstract

17 Cytokines are fundamental for a functioning immune system, and thus, potentially serve as  
18 important indicators of animal health. Quantitation of mRNA using quantitative reverse  
19 transcription polymerase chain reaction (qRT-PCR) is an established immunological technique.  
20 It is particularly suitable for detecting the expression of proteins against which monoclonal  
21 antibodies are not available. In this study, we developed a novel probe-based quantitative gene  
22 expression assay for immunological assessment of captive beluga whales  
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24 aquariums worldwide. Six immunologically relevant genes (IL-2R $\alpha$ , -4, -10, -12, TNF $\alpha$ , and  
25 IFN $\gamma$ ) were selected for analysis, and two validated housekeeping genes (PGK1 and RPL4) with  
26 stable expression were used as reference genes. Sixteen blood samples were obtained from four  
27 animals with different health conditions and stored in RNAlater solution. These samples were  
28 used for RNA extraction followed by qRT-PCR analysis. Analysis of gene transcripts was  
29 performed by relative quantitation using the comparative Cq method with the integration of  
30 amplification efficiency and two reference genes. The expression levels of each gene in the  
31 samples from clinically healthy animals were normally distributed. Transcript outliers for IL-  
32 2R $\alpha$ , IL-4, IL-12, TNF $\alpha$ , and IFN $\gamma$  were noticed in four samples collected from two clinically  
33 unhealthy animals. This assay has the potential to identify immune system deviation from normal  
34 state, which is caused by health problems. Furthermore, knowing the immune status of captive  
35 cetaceans could help both trainers and veterinarians in implementing preventive approaches prior  
36 to disease onset.

37

38 Keywords: qRT-PCR; Immunology; Gene expression; Beluga

## 40 Introduction

41 In recent years, there has been rapid development in the field of cetacean immunology,  
42 resulting in new methods to prevent and treat infectious diseases in captive animals being used in  
43 education programs and naval defense. Because the free-ranging cetaceans may serve as ideal  
44 sentinels of ecosystem health, efforts to develop reliable and relevant immunological techniques  
45 to address specific aspects of health and disease have increased (Beineke et al. 2004). Although  
46 cetacean species share many key immunological components with lab animals and humans,  
47 managing and evaluating the health of cetacean species remain a challenge (Sitt et al. 2008).  
48 Leukocyte transcriptional biomarkers such as cytokine genes have potential to assist in cetacean  
49 health assessment because of the broad scope of their functions and association with diseases  
50 known in human and veterinary medicine (Chaussabel 2015). Cytokines are important in  
51 regulating the initiation, maintenance, and amplification of the immune response. Therefore,  
52 monitoring and evaluating a set of cytokines expressed within a certain microenvironment can be  
53 a diagnostic tool for characterizing immune responses to foreign antigens and vaccines (de Jager  
54 et al. 2009). It can also identify perturbations of the immune system induced by environmental  
55 insults.

56 Cytokine gene transcripts from several cetacean species have been recently cloned, and their  
57 DNA sequences have been determined. Quantitative analyses of cetacean gene transcripts have  
58 been reported in beluga whales (*Delphinapterus leucas*), Pacific white-sided dolphins  
59 (*Lagenorhynchus obliquidens*), bottlenose dolphins (*Tursiops truncatus*), harbor porpoises  
60 (*Phocoena phocoena*), and killer whales (*Orcinus orca*) (Beineke et al. 2007; Buckman et al.  
61 2011; Mancina et al. 2008; Müller et al. 2013; Sitt et al. 2010; Sitt et al. 2008; Sitt et al. 2016).  
62 These studies were based on quantitative reverse transcription polymerase chain reaction (qRT-

63 PCR) using SYBR Green and various different house-keeping genes (HKGs). These studies  
64 enabled better understanding of the relative health of free-ranging cetacean species and *in vivo*  
65 baseline levels of gene expression in captive populations. Because of the short half-lives of  
66 leukocyte gene transcripts encoding surface and secreted messengers and their tight  
67 transcriptional control, identifying disease-specific or antigen-specific patterns of cytokine gene  
68 expression could facilitate animal health maintenance.

69 qRT-PCR is a sensitive method commonly used in both basic and diagnostic research to  
70 quantify mRNA level, and to provide real-time measures of blood leukocyte gene transcripts.  
71 However, only probe-based quantification methods offer minimum non-specific fluorescence  
72 and high sensitivity to detect a single gene transcript compared with other dye-based chemistries,  
73 enabling an accurate quantification of the amplified targets (Vanysacker et al. 2014). Therefore,  
74 probe-based qRT-PCR with validated reference genes is a preferred method for precisely  
75 quantifying mRNA abundance and detecting of small changes in gene expression (Wong et al.  
76 2015). Here we developed a novel species-specific probe-based qRT-PCR assay to measure the  
77 differential expression of immunologically relevant genes in beluga blood. Our findings could  
78 serve as a foundation for using transcriptional biomarkers for diagnosing diseases and assessing  
79 immunological profiles in captive and free-ranging cetaceans.

## 81 **Materials & Methods**

### 82 **Sample collection and preservation**

83 The voluntary blood collection of beluga in human care in the National Museum of Marine  
84 Biology and Aquarium was performed according to the international guidelines. Animal protocol  
85 was reviewed and approved by the Council of Agriculture of Taiwan (Approval number  
86 1020727724). Sixteen blood samples from four 14-year-old adult animals (females, A & C;  
87 males, B & D; samples from clinically healthy animals: A, 2 samples, B, 4; C, 2; D, 4; clinically  
88 unhealthy: A, 2; C, 2) were obtained on a monthly basis from 2013 to 2014. In 5 min after blood  
89 collection, 500  $\mu$ L of EDTA-anticoagulated whole blood was preserved by adding 1.3 mL  
90 RNeasy (Ambion, Applied Biosystems, Foster City, CA, USA). Samples were stored at  $-20^{\circ}\text{C}$   
91 until analysis. Samples from clinically healthy animals were used for establishing baseline values.

92

### 93 **RNA extraction and cDNA synthesis**

94 The RiboPure™-Blood Kit (Ambion) was used for total RNA isolation from blood samples  
95 according to the manufacturer's instructions. RNase inhibitor (RNA Armor™ Reagent, Protech,  
96 Taipei, Taiwan) was added to the RNA solutions to prevent RNA degradation. RNA integrity  
97 was routinely examined using denaturing gel electrophoresis. RNA concentration was measured  
98 using fluorometer assay [Qubit™ fluorometer with a Quant-iT™ RNA Assay Kit (Invitrogen,  
99 Carlsbad, CA, USA)]. Genomic DNA (gDNA) wipeout solution (Qiagen, Valencia, CA, USA)

100 was added in the RNA samples for gDNA removal, and gDNA contamination was confirmed by  
101 qPCR prior to adding reverse transcription reagents. For cDNA synthesis, 73–444 ng of RNA  
102 and reverse transcription kit (QuantiTect<sup>®</sup>, Qiagen) were used. Unused extracted RNA and  
103 cDNA were stored at –80 °C.

104

### 105 **Primer and probe design**

106 The Sequences of six immunologically relevant genes (IL-2R $\alpha$ , IL-4, IL-10, IL-12, IFN $\gamma$ , and  
107 TNF $\alpha$ ) of cetaceans were obtained from GenBank (Table 1). The overall aim was to measure the  
108 gene expression of the immune-related activities, including pro-inflammatory, Th1/Th2, T cell  
109 growth, and anti-inflammatory features. For the probe-based qRT-PCR assay, a web-based  
110 software (ProbeFinder, v.2.49, Roche, Pleasanton, CA, USA) was used for designing specific  
111 primers and corresponding probes (Universal ProbeLibrary, Roche) (Table 1). Primer specificity  
112 of the six genes was validated using PCR (Fast-Run Hotstart PCR kit, Protech) and  
113 electrophoresis. Two validated reference genes (PGK1 and RPL4) (Chen et al. 2016) were  
114 included for normalization.

115

### 116 **Quantitative PCR**

117 This study was conducted according to the MIQE guidelines (Bustin et al. 2009).  
118 Complementary DNA (cDNA) was analyzed by quantitative PCR using FastStart Essential DNA



119 Probes Master (Roche) according to the manufacturer's protocol. Thermal cycling was  
120 conducted using the Eco machine (Illumina, San Diego, CA, USA) and the same conditions were  
121 used for all target genes: 95°C for 10 min for polymerase activation, followed by 45 cycles of  
122 95°C for 10 s and 60°C for 30 s for denaturation and annealing/elongation, respectively. All  
123 reactions including plate controls and blank controls were run in triplicate. Plate controls include  
124 identical reaction materials on every run. A stable Cq value from all plate controls allowed data  
125 from multiple plates to be consolidated into a single data set. Threshold value for each candidate  
126 gene was manually set (Table 1). Baseline values were assigned for all plates using the Eco  
127 Software V4.0 (Illumina). PCR amplification efficiency (E) was calculated as  $E = (10^{(-1/\text{slope})} - 1)$   
128  $\times 100\%$ , where slope is the gradient of a standard curve. A gene-specific E for the following  
129 normalized value (NV) calculation was obtained from the average of at least three E values for  
130 each gene.

131

## 132 **Data analysis**

133 Analysis of qRT-PCR data was conducted using NVs modified from Pfaffl et al. (2002):  $\text{Log}_2$   
134  $(E_T^{CqT}/\text{Geomean}(E_{R1}^{CqR1} \& E_{R2}^{CqR2}))$ , where  $E_T$ ,  $E_{R1}$ , and  $E_{R2}$  are efficiencies of target gene and  
135 reference genes, and CqT, CqR1, and CqR2 are Cq values of target gene and reference genes.  
136 Lower NVs indicate higher target gene expression levels. Outliers are defined as values more  
137 than the third quartile +  $1.5 \times \text{IQR}$  or less than the first quartile -  $1.5 \times \text{IQR}$ , where IQR is the

138 interquartile range.

## 140 Results and Discussion

141 E values of all the six candidate genes ranged from 93.92% to 98.99% with  $R^2$  values of  
142  $>0.99$  (Table 1); therefore all six genes were included in the analysis of NVs. Figure 1 illustrates  
143 variable NV levels in the six candidate genes from clinically healthy samples with the lowest  
144 median NV (4.63) in IL-2R $\alpha$  and the highest (13.89) in IFN $\gamma$ . Gene transcript levels were used to  
145 establish three arbitrary categories: IL-2R $\alpha$  (highly transcribed; median NV  $< 5$ ), IL-10 and  
146 TNF $\alpha$  (moderately transcribed), and IFN $\gamma$ , IL-4 and IL-12 (lowly transcribed; (median NV  $> 8$ ).  
147 Shapiro–Wilk test showed that NV data of each gene was normally distributed ( $P > 0.05$ ).  
148 Stability in NVs during periods of health indicated the potential use in diagnostics by identifying  
149 outliers.

150 When pooling all samples (healthy and unhealthy) in one dataset, NV outliers in IL-2R $\alpha$ , IL-  
151 4, IL-12, IFN $\gamma$ , and TNF $\alpha$  in four samples (A-I, A-II, C-I, and C-II) from two clinically  
152 unhealthy individuals were identified. NV of IL-12 in sample A-I was 3.03, demonstrating that  
153 the transcript level of IL-12 was elevated. NV of IL-2R $\alpha$  in sample A-II was 3.45, revealing that  
154 the transcript level of IL-2R $\alpha$  was elevated. NV of IL-4 in sample C-I was 12.91, showing that  
155 the transcript level of IL-4 was decreased. NVs of IFN $\gamma$ , IL-2R $\alpha$ , IL-4, and TNF $\alpha$  in sample C-II  
156 were 7.91, 5.78, 11.45, and 15.50, revealing that the transcript level of IFN $\gamma$  in this sample was  
157 elevated, whereas those of other genes were decreased.

158 In this study, we selected a panel of genes to cover a wide range of immunological events in

159 beluga. The products of the selected genes are strong mediators of the immune system; they play  
160 a key role in the selection of immunological pathways and provide a link between innate and  
161 adaptive immune responses. IL-12 is a pro-inflammatory cytokine that induces proliferation and  
162 differentiation of T cells (Hsieh et al. 1993). TNF $\alpha$  is another pro-inflammatory cytokine that  
163 exerts cytotoxicity and induces cytokine secretion (Clark 2007). It can also restrict the local  
164 spreading of infection. The  $\alpha$  chain of IL-2 receptor is not expressed on resting T cells but only  
165 on activated T cells and is also called T cell activation (TAC) receptor (Liao et al. 2011). IFN $\gamma$  is  
166 produced by Th1 cells and shifts the response toward a Th1 phenotype (Schroder et al. 2004). IL-  
167 4 suppresses the production of Th1 cells and is required for the production of IgE (Sokol et al.  
168 2008). IL-10, an important immunoregulatory and anti-inflammatory cytokine (Mosser & Zhang  
169 2008), inhibits the synthesis of a number of cytokines involved in the inflammatory process,  
170 including IL-2, TNF $\alpha$  and IFN $\gamma$ . It is also a promotor of Th2 response via the suppression of IL-  
171 12 synthesis. Altogether, the selected genes could reflect the complexity of immunological  
172 responses, and their products represent valuable immunological markers.

173 IL-12 in blood is mainly produced by neutrophils and monocytes in response to pathogens  
174 (bacteria, fungi, intracellular parasites and viruses)(Trinchieri 2003). IL-2R $\alpha$  expression on  
175 leukocytes (neutrophils, NK cells, and activated T helper and regulatory cells) has been reported  
176 as a potential non-specific marker of an activated immune system (Zoldan et al. 2014). Beluga A  
177 had open wounds on mandible and several traumas on head, fins, and trunk when sample A-I and

178 A-II were collected, respectively (8-month difference between A-I and A-II collection date).  
179 Although the blood work and behavior of beluga A did not show obvious signs of infection, the  
180 higher expression levels of IL-12 in A-I and IL-2R $\alpha$  in A-II suggested that the immune system  
181 was activated as a result of wounds. Sample C-II was obtained when beluga C showed reluctance  
182 to the voluntary blood draw. Meanwhile, high fluke temperature was detected by infrared  
183 thermography, and vesicles were observed on the fluke of beluga C. Elevated expression levels  
184 of IFN $\gamma$  and decreased levels of TNF $\alpha$  in C-II were suggested as normal responses to social  
185 stressors in the environment, as previously observed in a killer whale study (Sitt et al. 2016).  
186 However, the possibility of an immune response from a virus infection could not be ruled out  
187 because of the clinical findings of the fluke. Sample C-I was taken when beluga C showed  
188 reluctance to the voluntary blood draw, and its blood work showed low serum iron level.  
189 Decreased expression levels of IL-4 in samples C-I and C-II were unexpected. More studies are  
190 required to clarify the function of IL-4 because it plays an important role in Th2 cell-mediated  
191 immunity, tissue repair, and homeostasis (Gadani et al. 2012).

192 Compared with solid organs, blood is a homogeneous tissue in which cellular composition  
193 can considerably vary depending on the location from where the sample is obtained. Without  
194 proper preservation, the copy number of individual mRNA transcripts in blood samples can  
195 change more than 1000-fold during storage and transport (Bowen et al. 2012). Two commonly  
196 used methods for stabilizing blood RNA are the PAXgene Blood RNA vacutainer tube and

197 RNAlater. These methods disrupt cells and precipitate RNA immediately upon homogenization  
198 by shaking the evacuated blood collection tubes. The collection tubes can then be stored frozen  
199 at  $-20^{\circ}\text{C}$  indefinitely without further processing. A previous study on the stability of RNA  
200 transcript from blood leukocytes using the above methods showed that both methods were  
201 suitable for use based on good quantity, integrity and purity of the isolated RNA (Weber et al.  
202 2010). In this study, we used RNAlater and smaller volume of blood (0.5 mL) to facilitate  
203 sample collection and transportation. Therefore, it was possible to adapt the methodology for  
204 serial sampling using small volumes of blood, which provides a temporal perspective  
205 transcriptome analysis.

206 Probe-based real-time assays with improved specificity are very useful in detecting low  
207 abundance cytokines for immunological research. The detection of cytokine mRNA using qRT-  
208 PCR has been suggested to be the only technique sensitive enough for reliable quantification *in*  
209 *vivo* (Huggett et al. 2005). The widely used SYBR Green assays in previous studies on cetacean  
210 gene transcripts have potential limitations such as primer-dimer formation, secondary structure  
211 formation by randomly binding to double stranded DNA, overestimate of target DNA, and  
212 higher inter-assay variation (Vanysacker et al. 2014). A fluorogenic probe-based approach with  
213 enhanced specificity was used in this study to prevent the limitations of SYBR Green assays.  
214 Amplification efficiency and reference gene selection are two important factors in gene transcript  
215 study using qPCR. The traditional NV is calculated using the  $\Delta\text{Cq}$  method ( $\text{CqT} - \text{CqR}$ ).

216 However, the traditional  $\Delta C_q$  method can overestimate the error, and the calculation of the gene  
217 expression level requires correction when the amplification efficiency is not close to 100%  
218 (doubling of PCR products per cycle)(Yuan et al. 2008). Moreover, using validated reference  
219 genes with stable gene transcript levels in varying experimental conditions can detect small  
220 perturbations with good sensitivity (Dheda et al. 2005). In this study, we determined the gene  
221 expression profiles by implementing rigorously calculated PCR amplification efficiency (E) and  
222 two validated reference genes. This is notably applicable to clinical sample with high variability  
223 and small changes in gene expression.

224 This study established a novel probe-based qRT-PCR assay for accurate and reliable detection  
225 and quantification of six immunologically relevant genes (IL-2R $\alpha$ , IL-4, IL-10, IL-12, IFN $\gamma$ , and  
226 TNF $\alpha$ ) and two validated reference genes (PGK1 and RPL4) in beluga. The real-time assay was  
227 successfully developed using a specific qRT-PCR protocol with the same chemistry and  
228 temperature profile, providing a simple and highly sensitive evaluation of normalized gene  
229 expression profiles. Preliminary data regarding the immune response of clinically unhealthy  
230 beluga serves as a reference for future studies characterizing a range of health conditions of  
231 beluga. This novel tool for evaluating peripheral blood cytokine gene expression levels in  
232 cetaceans would facilitate research on the immune response of animals in the marine habitat in  
233 response to environmental insults, as well as the etiology of infectious diseases or stress.

234

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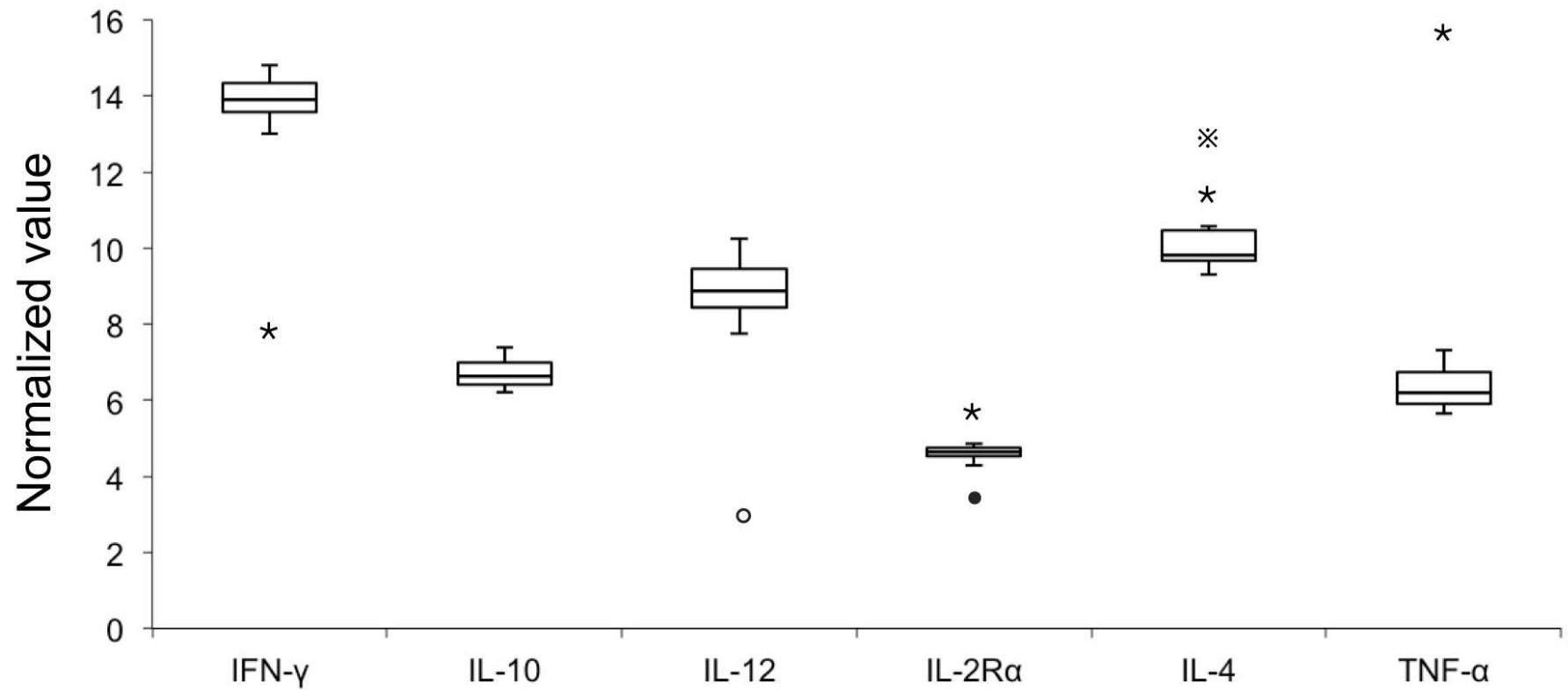
326 Table 1 Name, accession number, primer sequence, probe number, amplicon size, efficiency and R<sup>2</sup> of 6 immunologically relevant  
 327 genes.

Gene Name	Accession Number	Primer Sequence (5'-3')	UPL Probe Number	Amplicon Size (bp)	Threshold	Efficiency (%)± SD	R <sup>2</sup>
IL-2R $\alpha$	XM_00431350 1	F-TGAACCTTTGAAGAGAATTTACCA R-CTGAATCCCTGAATGCACTG	112	72	0.015	98.99±1.25	0.998
IL-4	NM_00128065 7.1	F-GCATGGAGCTGCCTGTAGA R-TGCAGAAAGTTTCCTTCTCAGTT	140	69	0.012	93.92±1.83	0.995
IL-10	AB775207.1	F-AAGCCCTGTCGGAGATGAT R-CACGTGCTCTTTGATGTTGG	25	86	0.012	97.25±3.61	0.995
IL-12	XM_00432440 2.1	F-CAGAAGGAGCTCTTTTATGACGA R-CCATGTGGTACATCTTCAAGTCC	98	71	0.015	93.95±3.04	0.997
TNF- $\alpha$	NM_00128061 5.1	F-CCAAGTGGCTACTCCATCATC R-CGGGCTTGTTACTTGAGGTT	106	76	0.012	94.44±2.00	0.997
IFN- $\gamma$	AB022044.2	F-TTTTCAGCTATGCGTGATTTTG R- TGCATTAAAATATTCCTTTAGGTTTTG	129	94	0.010	95.78±2.83	0.997

329 **Figure Legends**

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331 Figure 1 Box plot of normalized value (NV) of 6 immunologically relevant genes for blood samples from clinically healthy beluga  
332 (n=12) and outliers (n=4). Open circle: sample A-I; closed circle: sample A-II; note: sample C-I; asterisk: sample C-II.



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