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Short Communication

Development of a multiplex gene expression assay for components of the endocrine growth axis in coho salmon

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ABSTRACT

This study explores the efficacy of the Quantigene plex (QGP) technology for measuring a panel of endocrine growth-related transcripts in coho salmon, *Oncorhynchus kisutch*. The QGP technology permits the simultaneous quantification of multiple targeted mRNAs within a single tissue homogenate using sequence-specific probes and requires no reverse transcription (RT) or amplification as is required for RT-quantitative PCR (RT-qPCR). Using liver homogenates from coho salmon under fed and fasted conditions, we compared the detectable fold differences of steady-state mRNA levels between the QGP and probe-based RT-qPCR assays for insulin-like growth factors (*igf1* and *igf2*), insulin-like growth factor binding proteins (*igfbp1b*, *igfbp2a*, and *igfbp2b*), somatolactin receptor (*slr*), and growth hormone receptors (*ghr1* and *ghr2*). Significant, positive correlations for all genes between the two assays were found. In addition, the relatively low variance of results from the QGP assay suggests that this is a suitable method for a comprehensive analysis of endocrine growth-related transcripts and could potentially be used to develop assays for other gene networks in teleosts.

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1. Introduction

The endocrine growth axis is comprised of hormones, receptors and hormone binding proteins that function together to stimulate and modulate cellular growth and differentiation, ultimately regulating organismal growth (Kostyo and Goodman, 1999). A great deal has been learned about the mechanisms of this system by studying the individual components, including growth hormone (GH) and insulin-like growth factor 1 (IGF1) (Wood et al., 2005; Shimizu et al., 2009; Beckman, 2011). However, a broader and more complete understanding might be achieved by assessing multiple components of the axis simultaneously. Here we provide validation of a Quantigene multiplex (QGP) assay[1], capable of measuring levels of multiple mRNAs within a single tissue homogenate.

The QGP assay is a sandwich branched-chain DNA assay that utilizes cooperative hybridization to specifically capture target mRNA transcripts onto labeled xMAP beads (Flagella et al., 2006). Unlike reverse transcription-quantitative PCR (RT-qPCR) assays, the QGP assay is able to quantify mRNA by amplification of a fluorescent signal from a single mRNA copy instead of amplifying the target itself (Flagella et al., 2006). The QGP assay also allows for the direct quantification of mRNA from tissue homogenates, unlike RT-qPCR, which requires several intermediate steps that can increase variability between samples (Bustin, 2002). Quantitative PCR also has limited multiplexing capabilities and is sensitive to genomic DNA contamination (Bustin, 2002; Bustin and Nolan, 2004), which is not the case with QGP assays. The QGP assay has been used successfully in mammalian cell culture systems and on formalin-fixed, paraffin embedded tissues (Flagella et al., 2006; Knudsen et al., 2008). In fishes, a singleplex version of this assay (i.e., one mRNA target) is already being used in zebrafish to assess the effects of endocrine disrupting chemicals on aromatase gene expression (Hinfray et al., 2006; Cheshenko et al., 2007). However, no published reports have examined the efficacy of the multiplex QGP platform in teleosts.

The focus of this study was to validate a custom QGP assay designed to simultaneously measure eight different mRNAs (*igf1*, *igf2*, IGF binding protein 1b (*igfbp1b*), *igfbp2a*, *igfbp2b*, GH receptor 1 (*ghr1*), *ghr2*, and somatolactin receptor (*slr*)) involved in the endocrine growth axis of coho salmon (following nomenclature from Fukada et al., 2005; Raven et al., 2008; Shimizu et al., 2011a,b). Liver tissue was chosen for initial validation because it is one of the primary tissues involved in production of endocrine growth-related factors, including IGF1, IGF2, and the IGFBPs (Gray et al., 1992; Duan, 1997, 1998; Perez-Sanchez and Le Bail, 1999). Coho salmon were subjected to various fed, fasted and re-fed conditions to induce a large range of cellular transcript abundance to validate the gene expression assays. Sub-samples of liver tissue were then

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[1] Reference to trade name does not imply endorsement by the NMFS, NOAA.

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taken to compare differences in transcript level quantification between the RT-qPCR and QGP platforms.

2. Materials and methods

2.1. Fish – biological samples

One-year-old coho salmon (fork length 16.1 ± 1.3 cm (mean ± SD), body weight 48.7 ± 15.3 g, $n = 364$) were reared at the Northwest Fisheries Science Center in Seattle, WA, USA. They were maintained in recirculated fresh water in circular fiberglass tanks under incandescent light; flow rate was 25 L/min; temperature ranged from 10.5 °C to 13.0 °C; photoperiod was adjusted to that of Seattle, WA weekly (48°N). Fish were fed standard rations (0.6–1.0% body weight/day) of a commercial diet (Biodiet Grower; Bioproducts Inc., Warrenton, OR, USA) prior to sampling.

2.2. Experimental design and sampling procedure – biological samples

Samples from previously published work (Metzger et al., 2012; Shimizu et al., 2009) were used for this study. Briefly, one group of fish was fed a standard ration daily for 3 wk with the last meal provided 6 h prior to sampling, one group of fish was fasted for 3 wk and then provided a single meal 6 h prior to sampling, while the third group of fish was fasted for 3 wk and did not receive a meal prior to sampling. During scotophase, fish were netted using a dim red light and immediately killed in a solution containing tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA, USA). Livers were removed, weighed, and then a portion was immediately snap-frozen in liquid nitrogen. The experiment was conducted under an approved protocol of the University of Washington Institutional Animal Care and Use Committee (2313-09). These treatments resulted in a wide range of plasma levels of insulin, IGF1, IGFBP1b, IGFBP2b and GH (Shimizu et al., 2009) and were expected to result in a wide range of hepatic expression levels of components of the endocrine growth axis.

2.3. Technical variance samples

Livers were obtained from 3 yearling coho salmon (fork length: 159 ± 6 mm, body weight 45 ± 4 g) that had been fed daily to develop a dilution curve and test the technical variance of the QGP assay. Samples were collected 16 h after a meal was provided. Livers were weighed and then immediately snap frozen in liquid nitrogen.

2.4. Partial cDNA cloning and sequencing

PCR primers for cloning partial cDNAs for coho salmon *igfbp2a* and *igfbp2b* were designed in conserved sequence regions amongst salmonid fishes using MacVector version 9.5 software (Accelrys, San Diego, CA, USA). Primer sequences were as follows: *igfbp2a* Fwd GGCTTTCGTGGGAGCTTCTTTC, *igfbp2a* Rev TGTTTGAGGTTATACTGCCCCCTC, *igfbp2b* Fwd GCTGCGTTGTTATCCCACTGTAG, *igfbp2b* Rev ATCTCTGCCATCTGAAGGACGG. Partial cDNAs were amplified using GoTaq Green master mix (Promega, Madison, WI, USA) and standard PCR conditions. PCR products were verified by gel electrophoresis, purified with a QIAquick PCR Purification kit (Qiagen Inc., Valencia, CA, USA), and ligated into pCR 2.1 vector (TA cloning kit, Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Plasmids from positive clones were purified using the QIAprep Spin Miniprep kit (Qiagen) and sequenced with Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were cleaned using the CleanSEQ kit (Agencourt Bioscience Corporation, Beverly, MA, USA) and sequenced on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Partial coho salmon sequences were deposited in the GenBank public database for *igfbp2a* (GenBank ID: JQ765393) and *igfbp2b* (GenBank ID: JQ765394).

2.5. RNA isolation and cDNA synthesis

Total RNA was isolated from 26 to 40 mg pieces of liver tissue by homogenization in 1 ml of Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA). RNA samples were further purified as previously reported (Picha et al., 2008; Metzger et al., 2012) by mixing an equal volume of Plant RNA Isolation Aid solution (Ambion, Austin, TX, USA), incubating at room temperature for 10 min, and centrifuging the samples at 15,000×*g* for 15 min to eliminate glycogen that may interfere with spectrophotometric analysis. The supernatant containing the RNA was transferred to a new tube and RNA was precipitated with LiCl (2.5 M final concentration) by incubation at −20 °C for 30 min. Samples were centrifuged at 16,000×*g* for 20 min to pellet the RNA. RNA was resuspended in RNase-free water and treated with DNase I (DNA Free kit; Ambion, Austin, TX, USA) following the manufacturer's routine procedure to eliminate any genomic DNA. RNA samples had final A_{260}/A_{280} ratios of 2.06–2.12 and A_{260}/A_{230} ratios of 1.93–2.13 as determined by spectrophotometry (NanoDrop Technologies Inc., Rockland, DE, USA). Complementary DNA was synthesized in 20 µl reactions from 1 µg of total RNA using SuperScript II reverse transcriptase (Invitrogen) with random primers (Promega, Madison, WI, USA).

2.6. Quantitative RT-PCR assay procedures

Primers and probes for RT-qPCR assays are listed in Table 1. Original assays were designed using Primer Express version 1.5 software (Applied Biosystems) from previously published sequences for coho salmon *ghr1* and *ghr2* (Raven et al., 2008), while *igfbp2a* and *igfbp2b* RT-qPCR assays were designed from sequences generated in this study. Other primers and probes used in this study were previously published as follows; *igf1* (Pierce et al., 2003), *igf2* (Campbell et al., 2006), *igfbp1b* (Pierce et al., 2006; following nomenclature of Shimizu et al., 2011a), *slr* (Fukada et al., 2005), *ef1a* (Luckenbach et al., 2010). All primers and probes were ordered from IDT (Coralville, IA, USA). Probes were designed with the 6FAM reporter and black hole quencher-1 (BHQ-1). Assay specificity was confirmed for all original RT-qPCRs by cloning and sequencing the amplicons. Briefly, RT-qPCR products were purified using Qiagen's PCR Purification kit and cloning and sequencing reactions were carried out as described above. Three separate clones from each assay were selected for sequencing to confirm the specificity of the assays distinguishing between isoforms of similar gene transcripts.

Quantitative PCRs consisted of 1× Taqman master mix (Applied Biosystems), 900 nM forward and reverse primers, 250 nM probe, and 0.1 ng cDNA template (based on RNA loaded into the RT reactions). No amplification controls (no reverse transcriptase was loaded in the RT reactions) and no template controls (water was used in place of template in the RT reactions) were included in each assay and showed no detectable amplification. Assays were run on an ABI 7700 Sequence Detection System in 96-well plates using the following cycling conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Complementary DNAs for a dilution curve were generated by pooling RNA from three individuals to use as a template in the RT reaction. Complementary DNA generated from pooled RNA template was serially diluted and run in duplicate in each assay at concentrations of 1, 0.2, 0.1, 0.02, 0.01, 0.002, 0.001, and 0.0002 ng. Standard curves were generated from the log input of the cDNA versus cycle threshold. Technical variance samples were