

Chapter 21

Quantification of Interferon Signaling in Avian Cells

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Abstract

Activation of the type I interferon (IFN) response is an essential defense mechanism against invading pathogens such as viruses. This chapter describes two protocols to quantify activation of the chicken IFN response through analysis of gene expression by real-time quantitative PCR and by quantification of bioactive IFN protein using a bioassay.

Key words Interferon, Bioassay, Chicken, Real-time quantitative PCR

1 Introduction

The type I interferon response (IFN response) is an important part of the immune reaction against viruses. Interferon alpha and beta (IFN α and IFN β) are the prototypical type I interferons and can be produced by most animal cells. Production of IFN α/β is triggered upon stimulation of pattern recognition receptors, such as Toll like receptors (TLRs) or Rig-I like receptors (RLRs). Upon production, IFN α and IFN β are rapidly secreted to the extracellular compartment, where they can bind to the ubiquitously expressed IFN receptor. Binding of IFN to the receptor activates the JAK/STAT signaling pathway, leading to the formation of the ISGF3 transcription complex consisting of a STAT1, STAT2, and IRF9. In the nucleus, the ISGF3 complex induces transcription of hundreds of IFN-stimulated genes (ISGs) [1]. Many of these genes encode proteins that interfere with the replicative cycle of viruses at various stages (reviewed in ref. 2). The IFN response is a potent antiviral mechanism, and therefore, most viruses have been evolutionarily selected to counteract it and coronaviruses are no exception (reviewed by Zhong et al. [3]).

In this chapter we describe two protocols to quantify activation of the IFN response. We have found these protocols useful to study if and how viruses counteract the IFN response in chicken cells. The first protocol describes how to quantify activation of the

IFN response at the transcriptional level using real-time quantitative PCR (RT-qPCR) on *Ifn* and IFN-stimulated genes. The second protocol describes quantification of bioactive type-I IFN protein (both IFN α and IFN β) by the use of a reporter cell line. This bioassay can be used to quantify IFN secreted in response to virus infection and, when combined with transcription analysis of *Ifn α* and *Ifn β* these assays can provide an integral picture of activation of the chicken IFN response.

1.1 Quantitation of Transcription of Chicken *Ifn*-Related Genes

Similar to most mammalian cell lines, activation of the interferon response in most chicken cells is characterized by upregulation of *Ifn β* . Like the human genome, the chicken genome encodes only one copy of the *Ifn β* gene, whereas at least ten isoforms of *Ifn α* are present [4, 5]. Similar to mammals, production of chicken IFN α is mainly mediated by monocytes; other cells mainly produce IFN β in response to viral infection [6]. Because avian coronaviruses replicate mainly in epithelial cells, we monitor activation of the type I interferon response by quantification of *Ifn β* . Similar to mammalian cells, *Ifn β* is upregulated upon activation of either TLR or Rig-I like receptors (RLRs), but not in response to stimulation with IFN. Concomitant with *Ifn β* , many ISGs are also upregulated, indicating that the term interferon stimulated genes is somewhat misleading [7]. Studying the expression of ISGs can be useful, therefore we have provided a list of avian-specific primers for use in real-time quantitative PCR (RT-qPCR; Table 1) [8–10]. Protocols for RNA isolation, cDNA synthesis and real-time PCR are plenty and every lab has its own protocols. In this chapter we describe briefly the methods used in our lab. For a detailed overview of RT-qPCR techniques and theoretical background, please refer to Forlenza et al. [11].

1.2 Quantitation of Chicken Type I IFN Protein Using a Bioassay

The chicken interferon bioassay was developed in the laboratory of Prof. P. Stacheli [12]. It is based on a quail cell line (CEC-32) that contains the luciferase gene downstream of a part of the inducible chicken *mx* promoter. Stimulation of these cells with type I interferon readily induces activation of the *mx* promoter and subsequent production of the firefly luciferase enzyme. Firefly luciferase can be easily quantified using commercially available luciferase assay kits. Here we provide a step-by-step protocol for measuring IFN concentrations using this bioassay.

2 Materials

2.1 RNA Isolation, cDNA Synthesis, and RT-qPCR on Avian Cells

1. Cells and virus, as per experiment.
2. RNeasy Mini Kit (QIAGEN).
3. RNase-free DNase set (QIAGEN).

Table 1

Chicken-specific real-time qPCR primers, including accession numbers of the sequences used to design the primers

gene	sense	sequence (5'-3')	Accession nr.	reference
IFN β	FW	GCTCTCACCACCACCTTCTC	ENSGALT00000039477	
	RV	GCTTGCTTCTTGTCCTTGCT		
IFN α	FW	ATCCTGCTGCTCAGCTCCTTCT	XM_004937096	8
	RV	GGTGTGCTGGTGTCCAGGATG		
IRF3	FW	CAGTGCTTCTCCAGCACAAA	NM_205372	
	RV	TGCATGTGGTATTGCTCGAT		
IRF1	FW	CAGGAAGTGGAGGTGGAGAA	ENSGALG00000006785	
	RV	TGGTAGATGTCGTTGGTGCT		
TLR7	FW	TTCTGGCCACAGATGTGACC	NM_001011688	8
	RV	CCTTCAACTGGCAGTGCAG		
TLR3	FW	TCAGTACATTTGTAACACCCCGCC	NM_001011691	8
	RV	GGCGTCATAATCAAACACTCC		
MDA5	FW	TGGAGCTGGGCATCTTTCAG	GU570144	
	RV	GTTCCCACGACTCTCAATAACAGT		
Mx	FW	TTGTCTGGTGTGCTCTTCCT	ENSGALT00000025999	
	RV	GCTGTATTTCTGTGTTGCGGTA		
OAS	FW	CACGGCCTCTTCTACGACA	NM_205041	9
	RV	TGGGCCATACGGTGTAGACT		
IL8	FW	TTGGAAGCCACTTCAGTCAGAC	NM_205498	9
	RV	GGAGCAGGAGGAATTACCAGTT		
PKR	FW	CCTCTGCTGGCCTTACTGTCA	NM_204487	10
	RV	AAGAGAGGCAGAAGGAATAATTTGCC		
ADAR	FW	TGTTTGTGATGGCTGTTGAG	AF403114	
	RV	AGATGTGAAGTCCGTGTTG		
ISG12	FW	TAAGGGATGGATGGCGAAG	NM_001002856	
	RV	GCAATATCTTTATTGTTCTCAC		
MHC-I	FW	CTTCATTGCCTTCGACAAAG	NM_001031338	9
	RV	GCCACTCCACGCAGGT		
IFNAR2	FW	GCTTGTGTTTCGTCAGCATT	ENSGALT00000036778	9
	RV	TTCGCAATCTTCCAGTTGT		
Firefly Luciferase	FW	TGTTGGGCGCGTTATTTATC	X65316	
	RV	AGGCTGCGAAATGTTCATACT		

4. Bioanalyser (Agilent Technologies) or agarose gel electrophoresis equipment.
5. Spectrophotometer (NanoDrop or equivalent).
6. DNase I, Amplification Grade.
7. Reverse Transcriptase (Invitrogen SuperScript® III or equivalent).
8. PCR machine (for cDNA synthesis).
9. Nuclease-free water.
10. Luciferase mRNA.
11. Random hexamers.
12. 2× SYBR® Green I mix.
13. Quantitative-PCR machine (Qiagen Rotor-Gene Q or equivalent).
14. Primers (Table 1).

**2.2 Quantitation
of Chicken Type I IFN
Protein Using
a Bioassay**

1. Culture medium: DMEM, 10 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin.
2. Stimulation medium: DMEM, 1 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin.
3. CEC-32 chicken IFN reporter cells in 96-well plates at 70–90 % confluency (provided by P. Staeheli, *see* **Note 1**).
4. Recombinant chicken interferon alpha (chIFNα; Labome).
5. Multichannel pipet (8 × 200 µl).
6. Firefly luciferase assay buffer (Promega Bright-Glo™ or equivalent).
7. Luminometer.

3 Methods

**3.1 RNA Isolation,
cDNA Synthesis,
and RT-qPCR on Avian
Cells**

1. Perform the experiment in 24-well plates (*see* **Note 2**). Infect or treat cells as desired.
2. When appropriate, cells are lysed by adding 350 µl RLT buffer spiked with 1 ng/sample of luciferase mRNA prior to RNA isolation (*see* **Note 3**).
3. Total RNA is isolated using the RNeasy Mini Kit according to the manufacturer's instructions, including an on-column DNase treatment with RNase-free DNase.
4. Verify RNA integrity on a 1 % agarose gel or using a Bioanalyser.
5. Determine RNA concentration using a spectrophotometer (NanoDrop or equivalent).

6. Prior to cDNA synthesis, perform a second DNase digestion step using DNase I.
7. Synthesis of cDNA is performed on 0.5–1 µg total RNA using Reverse transcriptase and random hexamers according to the manufacturer's instructions. Incubation steps are performed in a regular PCR machine or, alternatively in a water bath.
8. After cDNA synthesis, samples are diluted 1:50 in nuclease-free water before qPCR analysis.
9. Per sample, prepare a master mix containing 7 µl 2× SYBR Green I Mix and 2 µl primer mix (2.1 µM forward and reverse primer).
10. Combine 9 µl master mix and 5 µl diluted cDNA per PCR tube.
11. Real-time quantitative PCR is performed on a qPCR machine, such as Rotor-Gene Q, 35–40 cycles, 60 °C annealing temperature, 20 s extension time.
12. Cycle thresholds and amplification efficiencies are calculated using the software pertaining to the qPCR machine, such as Rotor-Gene 6000.
13. Using Eq. 1, the relative expression ratio of the target gene is calculated using the average reaction efficiency for each primer set and the cycle threshold (C_t) deviation of sample vs. control at time point 0 h (*see Note 4*).

$$R_{\text{Ifn}\beta} = \frac{E_{\text{Ifn}\beta}^{(C_t^{\text{Ifn}\beta}(\text{calibrator}) - C_t^{\text{Ifn}\beta}(\text{sample}))}}{E_{\text{GAPDH}}^{(C_t^{\text{GAPDH}}(\text{calibrator}) - C_t^{\text{GAPDH}}(\text{sample}))}} \quad (1)$$

With:

R = fold change of the target gene relative to the control

Calibrator = control cells at time point 0 (zero)

E = average amplification efficiency for that set of primers

C_t = cycle threshold

3.2 Quantification of Chicken Type I IFN Protein Using a Bioassay

1. If the samples contain virus, heat-inactivate at 56 °C for 30 min prior to performing the assay. This treatment inactivates coronaviruses but retains bioactivity of type I IFN (*see Note 5*).
2. Fill a sterile 96-well plate with 50 µl stimulation medium/well.
3. Add 50 µl chIFN α standard (50 U/ml) or test sample to the first row (vortex before adding).
4. Make serial twofold dilutions in the plate using a multichannel pipet (Fig. 1, *see Note 6*).
5. Remove the medium from the CEC-32 cells which have been cultured in the 96-well plate (*see Note 7*).
6. Transfer the content of the plate containing the diluted samples and standard to the CEC-32 cells (*see Note 8*).

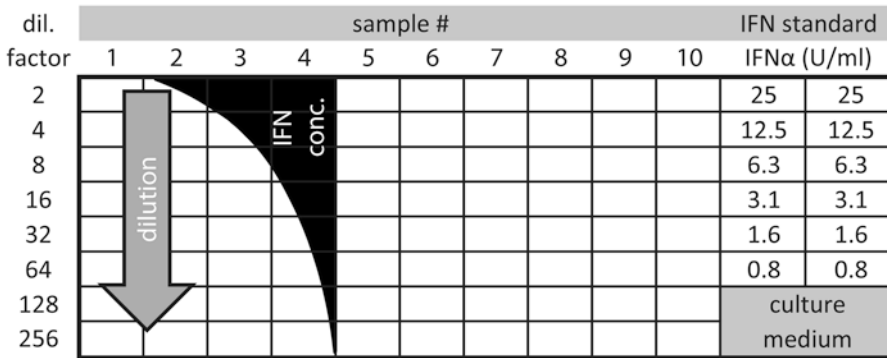


Fig. 1 Layout of a 96-well plate to accommodate ten samples and an interferon standard

7. Incubate plates at 37 °C and 5 % CO₂ for 6 h.
8. Use a firefly luciferase assay kit to detect luciferase activity, according to manufacturer’s instructions.

3.3 Calculation of IFN Concentration from Luminescence Data

To calculate the units of interferon in the original sample, a workflow is provided in Fig. 2.

1. Transfer the measurements from the luminometer to a spreadsheet program (Microsoft Excel or equivalent).
2. Calculate the average value of the background luminescence and subtract this value from all wells (Fig. 2, point A).
3. Calculate the average of the wells incubated with the diluted interferon standard and plot them in a scatter plot. This graph is the standard curve (B).
4. Make a new graph using only the data points that fall within the linear range of the standard curve, usually 1–12.5 or 1–6 U/ml.
5. Plot a linear trend line through these data points and display the equation on the chart (C).
6. Next, all luminescence values that fall within the linear range of the standard curve are selected (here 2–12 U/ml).
7. Calculate the IFN concentration in each well using the equation from the standard curve (D).
8. Multiply by the dilution factor to obtain the concentration of IFN in the undiluted samples (E).
9. Finally, calculate the average IFN concentration of the wells that fall within the linear range (usually two or three wells per sample). This value corresponds to the final concentration of type I interferon in the original sample (F).

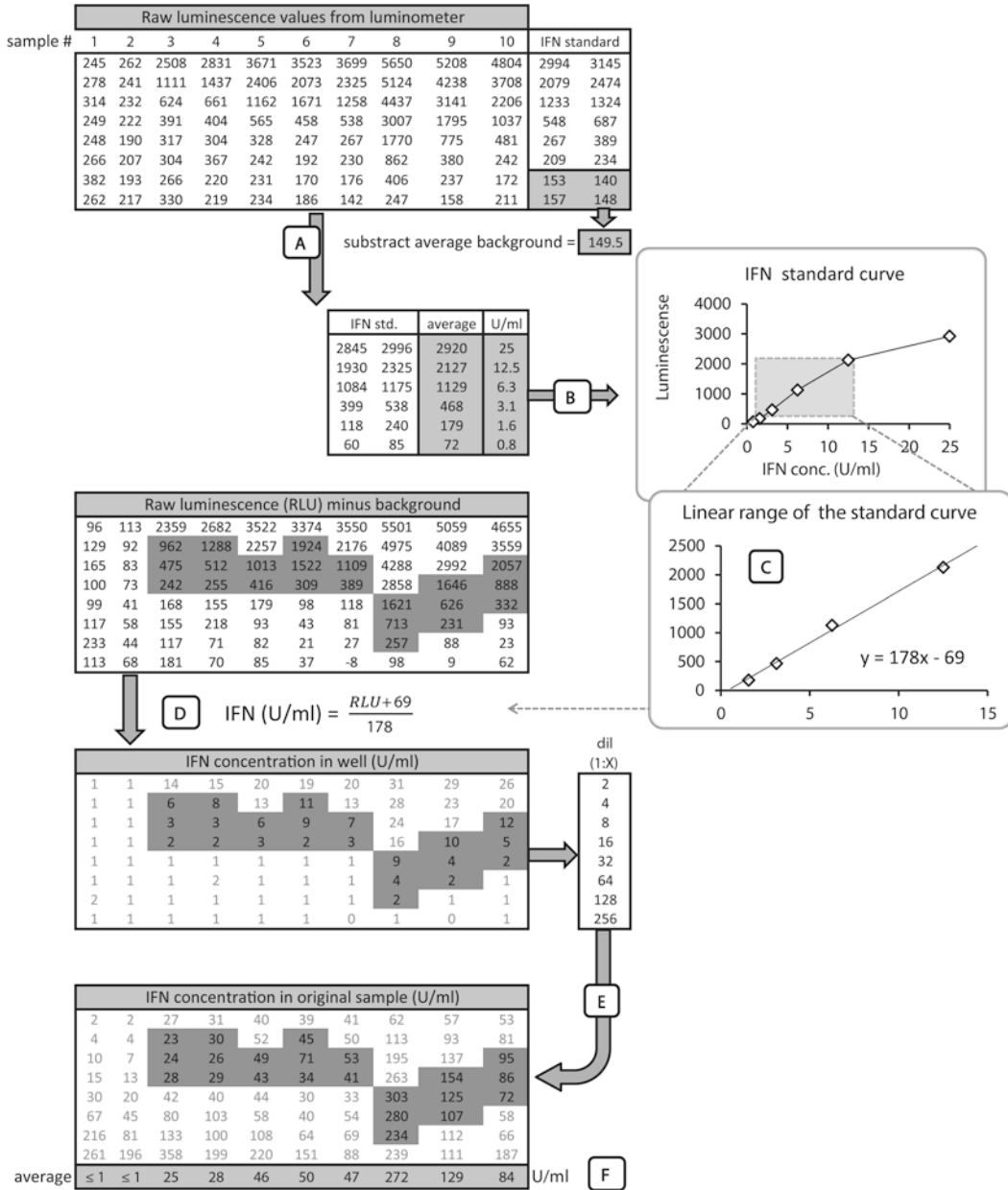


Fig. 2 Workflow on how to calculate the concentration of IFN in the original sample from the readout of the luminometer

4 Notes

1. For more detail on the construction of CEC-32 chicken-IFN reporter cells, *see* ref. 12.
2. To have enough RNA, each well of a 24-well plate should contain around 3×10^5 avian cells.

3. For normalization, a housekeeping gene such as GAPDH is generally used. It is advised to ensure that the reference gene selected is stable under the conditions of each experiment by performing stability analysis. When the mRNA level of the reference gene is not stable during the experimental procedure, such as during prolonged infection with a virus, we use an external reference gene for normalization. The external reference gene we use is luciferase, which is added as mRNA (commercially available) to the RLT lysis buffer (1 ng/sample) prior to RNA isolation and cell lysis. This guarantees that the external reference RNA and the host RNA are subject to the same treatment prior to cDNA synthesis.
4. To calculate the fold change of IBV total RNA, C_t deviation is calculated versus a fixed C_t value (e.g., $C_t = 30$), because no IBV is present in the non-infected cells that are used as control in all the experiments.
5. Interferon containing samples can be stored at 4 °C overnight. Storage at -20 °C ensures long time (>months) stability. One freeze-thaw cycle can reduce the IFN-activity of a sample by 40 %. To avoid repeated freeze-thawing of samples, avoid measuring the same sample twice. To achieve this, ensure that at least one of the dilutions of the samples falls within the linear range of the standard curve.
6. To select the appropriate dilutions it is advisable to perform a pilot experiment. One can either make an extensive twofold serial dilution series (for example, 2-1,024 times dilution), or use tenfold pre-dilutions. In our hands IFN production by avian cells rarely exceeds 5,000 U/ml, and therefore, a maximum of 1,000 times pre-dilution should suffice.
7. Medium is removed simply by emptying the 96-well plate in the waste and gently tapping it dry on a stack of tissues. Sterility is not an issue, the cells will only be incubated for another 6 h.
8. CEC-32 cells should *not be allowed to dry out!* Make sure you transfer the samples to the cells within minutes. Drying of the monolayer will decrease the luminescence and thereby negatively influence the assay. When transferring the diluted samples to 96-well plate with CEC-32 cells, start with the lowest concentration and work your way up the dilutions. In this way the same tips can be used for multiple dilutions. Do not forget to add medium to the negative controls.

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