Chapter 9

Monitoring the Transcriptional Activity of FOXO Transcription Factors by Analyzing their Target Genes

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Abstract

FOXO proteins represent a subfamily of transcription factors that belong to the forkhead family. The study of FOXO target genes can be performed using Real-Time PCR (RT-PCR). The RT-PCR is a sensitive method that allows the detection and quantification of minute amounts of nucleic acids. In RT-PCR the accumulation of the amplicon is detected and measured as the reaction progresses. Here, we describe the application of RT-PCR technique to monitor the transcriptional activity of FOXO transcription factors.

Key words FOXO, Transcription factor, Target genes, Real-time PCR, Fluorescent reporter, Quantitative

1 Introduction

FOXO proteins represent a subfamily of transcription factors that belong to the forkhead family, first described in *Drosophila* [1, 2]. In mammals there are four *FOXO* genes: *FOXO1*, *FOXO3*, *FOXO4*, and *FOXO6*, involved in crucial cellular processes like regulation of stress resistance, metabolism, cell cycle arrest, and apoptosis [3–6]. The control of several cellular processes requires the activation and repression of target genes, carried out in a large part by transcription factors [7]. The study of signaling networks regulated by FOXO transcription factors is essential to understand its functions. One of the first steps to identify target genes of a given transcription factor includes the overexpression or the knockdown of the transcription factor in study and the analysis of the resulting changes in gene expression [8]. Several *FOXO* target genes are already identified: *PTEN* [9], *p21* [10], *p27* [11], *FasL* [12], *TRAIL* [13], *CyclinD1* [14], *PUMA* [15], *Cited2* [16], or *BAX* [17]. Real-Time Polymerase Chain Reaction (RT-PCR) can be used to analyze gene expression of FOXO target genes. RT-PCR

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was first described in 1993 by Higuchi R. et al., where they describe the detection of the amplicon using a DNA-binding dye Ethidium Bromide (EtBr) [18]. RT-PCR proved to be a powerful tool to quantify gene expression and is considered the gold-standard method to analyze gene expression [19]. The difference between RT-PCR and regular PCR relies, mainly, on the fact that in PCR the amplified DNA product is detected in the end-point analysis, and in RT-PCR the accumulation of the amplification product is measured in real time, as the reaction progresses with the product quantification after each cycle [20]. The RT-PCR includes several processes. First, the RNA is isolated from the tissue/cell samples and transcribed into a complementary DNA (cDNA) by reverse transcriptase from mRNA. Then, cDNA is used as a template for the RT-PCR reaction. The reaction is detected by the use of fluorescent reporters that enables the detection of the amplicon as the reaction progresses [20]. The amount of amplification product in each cycle is calculated by the measurement of the increase in the fluorescent signal (from DNA-binding fluorophores like SyberGreen) [21–23]. The amount of the amplification product is given by the threshold cycle (Ct) in a PCR reaction. The Ct can be defined as the cycle number in which the fluorescence emission exceeds a chosen threshold (detectable signal is achieved) [21, 22]. The higher the mRNA concentration, the lower the Ct [22]. RT-PCR is an extremely accurate, fast, cost-effective method and allows qualitative and quantitative analysis [19, 24]. The chapter that follows describes a RT-PCR protocol to analyze the expression of FOXO target genes, using comparative Ct method ($2^{-\Delta\Delta Ct}$) [25].

2 Materials

Benches and material should be clean using RNase*Zap*. Use RNase and DNase-free pipettes and pipettes tips (filtered) and gloves. Waste material should be disposed according to waste disposal regulations (*see* **Note 1**).

2.1 RNA Extraction Using E.Z.N.A. Total RNA Kit

1. RNase*Zap*.
2. TRK Lysis Buffer: add 20 μL of 2-mercaptoethanol (2-mercaptoethanol should be stored in the refrigerator and used only in the extraction hood) per 1 mL of TRK Lysis Buffer.
3. 70% Ethanol prepared with RNase-free water.
4. DNAse (NZYtech): keep it on ice. Prepare DNAse in a DNase and RNase-free eppendorf: ~80 μL of DNase buffer per sample, add DNase.
5. RNA Wash Buffer I.
6. RNA Wash Buffer II.

7. RNase-free water.
8. Ice.
9. HiBind® RNA spin columns.
10. Vortex.
11. Centrifuge.
12. Sterile RNase-free pipette tips and microcentrifuge tubes.

2.1.1 RNA Quantification and Storage

1. NanoDrop ND-2000.
2. RNase and DNase-free pipettes and pipette tips.
3. RNase-free water.

2.2 cDNA Synthesis Using cDNA Synthesis Kit, NZYTech

IMPORTANT: Keep all the reagents on ice.

1. NZYRT Enzyme Mix (Includes NZY Reverse Transcriptase and NZY Ribonuclease Inhibitor).
2. NZYRT 2× master mix (includes oligo(dT)18, random hexamers, MgCl2, and dNTPs).
3. NZY RNase H (*E.coli*)..
4. DNase and RNase-free water.
5. Thermal Cycler.

2.3 Real-Time PCR Using LuminoCt Syber Green (Sigma Aldrich)

1. Ice box.
2. DNase and RNase-free PCR tubes.
3. DNase and RNase-free water.
4. Primers Forward and Reverse for each gene to be analyze (Table 1).
5. cDNA.
6. DNase and RNase-free PCR tubes.
7. DNase and RNase-free pipettes and pipette tips.
8. Plates and optical caps for specific thermal cycler.

Table 1
Primer sequences for real-time PCR

Gene of interest	Sequence (5′ → 3′)	
BAX	Forward	GTGGCAGCTGACATGTTTTC
	Reverse	GGAGGAAGTCCAATGTCCAG
p27	Forward	CCGGCTAACTCTGAGGACAC
	Reverse	CTTCTGAGGCCAGGCTTCTT
TRAIL	Forward	TTCACAGTGCTCCTGCAGTC
	Reverse	ACGGAGTTGCCACTTGACTT

9. Real-time quantitative thermal cycler.
10. LuminoCt SYBR Green qPCR ReadyMix.
11. Centrifuge.

3 Methods

3.1 RNA Extraction Using E.Z.N.A. Total RNA Kit

All procedures must be performed on a clean workspace. Before starting, carefully wipe the workspace and the material with RNaseZap (solution that destroys RNases on contact).

1. Add TRK Lysis Buffer (mix 20 μL of 2-mercaptoethanol in 1 mL of TRK Lysis Buffer) to each sample to lyse cells or tissues. The volume of TRK Lysis Buffer to be added depends on the sample size. If the number of cells is $\leq 5 \times 10^6$ add 350 μL, or 700 μL if the cell number is $\geq 5 \times 10^6$. Mix carefully with a 200 μL pipette using up and down movements.
2. Add the same volume of 70% ethanol to each sample (according to the volume used in **step 1**) and vortex. A precipitate might appear after adding ethanol, vortex again.
3. Transfer the entire sample to a HiBind® RNA spin column, with the spin column inside a 2 mL collection tube. The maximum capacity for this column is 750 μL. If the volume is higher you must repeat the procedure (**step 3**). Spin the samples at 10,000 × *g* for 30–60 s at room temperature. Discard the supernatant and the collection tube in the extraction hood (remember that TRK Lysis Buffer contains 2-mercaptoethanol).
4. Place a new 2 mL collection tube in each spin column. Add 250 μL of RNA wash buffer I to each column. Spin the samples at 10,000 × *g* for 30–60 s. Discard the supernatant and the collection tube. Place a new 2 mL collection tube in each spin column.
5. DNase treatment (*see* **Note 2**). Important: DNase must always be on ice. Prepare DNase solution: in RNase-free microcentrifuge tubes add 70 μL of DNase buffer and 10 μL of DNase per sample. Add 80 μL of the solution to each sample (*see* **Note 3**). Incubate for 15 min.
6. Add 500 μL of RNA Wash Buffer I. Spin the samples at 10,000 × *g* for 30–60 s. Discard the supernatant but do not discard the collection tube.
7. Add 500 μL of RNA Wash Buffer II (must be diluted with absolute ethanol before use). Spin the samples at 10,000 × *g* for 30–60 s. Discard the supernatant and keep the collection tube for **step 8** (*see* **Note 4**).
8. Repeat **step 7**.

9. RNA elution. Transfer the columns to sterile RNase-free microcentrifuge tubes. Elute the RNA with 50–100 μL RNase-free water. Centrifuge for 1 min at the maximum speed.

3.1.1 RNA Quantification and Storage

Eluted RNA (in RNase-free water or in an elution buffer such as TE) can be quantified using a Nanodrop2000 or a spectrophotometer. During these procedures always use clean gloves to avoid contaminations. Nucleic acids purity is measured by two ratios: absorption at 260–280 nm (A260/A280) and absorption at 260–230 nm (A260/A230). A260/A280 ratios below 1.8 are indicative of phenol contamination or low concentration of the nucleic acids while a low 260/230 ratio, below 2, indicates EDTA or carbohydrates contaminations, or residual phenol.

1. Wipe the Nanodrop2000 pedestal with a clean and dry paper.
2. Select RNA as the sample type. Pipette 1 μL of RNase-free water (blank, solution where RNA is diluted). Click on blank to calibrate the Nanodrop2000 for your samples measurements.
3. Wipe the pedestal. Pipette 1 μL of your sample and click measure. The software will measure the RNA concentration and the two ratios 260/280 and 260/230 (*see* **Note 5**).
4. Repeat **step 3** for the other samples. Wipe the Nanodrop2000 pedestal with a clean and dry paper.
5. RNA samples must be storage at −80 °C since RNA is highly unstable. Avoid multiple freezing and thawing cycles.

3.2 cDNA Synthesis Using cDNA Synthesis Kit, NZYTech

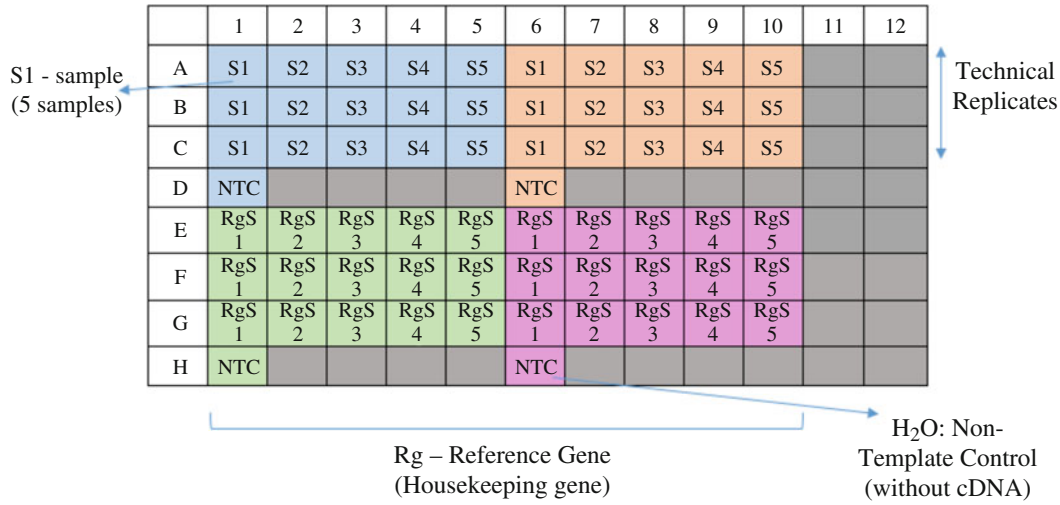
The following procedures must be performed on ice. Use clean gloves.

1. Prepare and label nuclease-free PCR tubes. Include 2 tubes for negative control (*see* **Note 6**).
2. Each sample requires 20 μL of reaction. Pipette for each sample tube: 10 μL of master mix, 2 μL of enzyme, 2 μg of RNA sample, and DNase and RNase-free water up to 20 μL (*see* **Note 7**).
3. Program the thermo cycler with the following protocol: 25 °C for 10 min, 50 °C for 30 min, and 85 °C for 5 min. Add 1 μL of RNaseH to each sample tube (*see* **Note 8**) and continue the reaction at 37 °C for 20 min. Add a final step at 4 °C to keep your samples refrigerated.
4. Dilute part of your cDNA in DNase and RNase-free water to tenfold (*see* **Note 9**). Keep part of your original cDNA stock, and freeze both (diluted and original stock of CDNA) at −20 °C (*see* **Note 10**).

3.3 Real-Time PCR Using LuminoCt Syber Green (Sigma Aldrich)

RT-PCR or q-PCR is a quantitative method that allows the analysis of gene expression. Depending on the total number of reactions (considering the number of your samples and the number of genes to analyze) you can use 96 or 384-well PCR plates (*see* **Note 11**). We recommend to perform at least 3 technical replicates and include in the analysis 2 or 3 housekeeping genes (genes constitutively expressed in a tissue) (*see* **Note 12**).

1. Prepare and label DNase and RNase-free PCR tubes for each gene/sample and for each of the master mixes (per gene). For each gene analyzed include a negative control containing no cDNA (non-template control). Make the calculations of each reagent needed (*see* **Note 13**).
2. Each reaction has 10 μL of total volume. Pipette the correct water volume to each pool master mix of each gene: 3,2 μL of water per reaction. Select the adequate oligos (oligos must be diluted at 10 μM). Place them on ice. Pipette the correct volume of primers to the corresponding pool master mix, final concentration 0,4 μM (*see* **Note 14**).
3. Thaw cDNA samples on ice.
4. Prepare the PCR plate according to the planned layout (example in Fig. 1).
5. Pipette 5 μL LuminoCt per each reaction to the pool master mix.
6. From each gene master mix pipette 9 μL to each sample well.



	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	S4	S5	S1	S2	S3	S4	S5		
B	S1	S2	S3	S4	S5	S1	S2	S3	S4	S5		
C	S1	S2	S3	S4	S5	S1	S2	S3	S4	S5		
D	NTC					NTC						
E	RgS 1	RgS 2	RgS 3	RgS 4	RgS 5	RgS 1	RgS 2	RgS 3	RgS 4	RgS 5		
F	RgS 1	RgS 2	RgS 3	RgS 4	RgS 5	RgS 1	RgS 2	RgS 3	RgS 4	RgS 5		
G	RgS 1	RgS 2	RgS 3	RgS 4	RgS 5	RgS 1	RgS 2	RgS 3	RgS 4	RgS 5		
H	NTC					NTC						

Fig. 1 Representation of a 96 well plate layout. Different genes are represented in different colors. Each gene is being assessed in five different samples using three technical replicates. The two housekeeping genes are represented in green and pink and labeled as Rg. NTC refers to the non-template control that should be included for each gene that is being analyzed

7. Pipette 1 μL of cDNA (diluted 1:10) to each correspondent well (*see* **Note 15**).
8. Seal the PCR plate using optical caps for specific thermal cycler (*see* **Note 16**).
9. Spin the PCR plate.

3.3.1 Program Setup

Bio-Rad CFX Manager is a program used for the detection of PCR and Real-Time PCR.

1. Start the CFX Manager program.
2. Select a protocol and click the EDIT button and define the protocol accordingly: 20 s at 94 °C, 3 s at 94 °C for denaturation, 20 s at 56 °C for annealing/extension and the final step at 4 °C. Select the number of cycles: 40.
3. Open the thermal cycler lid and place the PCR plate.
4. Close the lid and click RUN.

3.3.2 Data Analysis

Data can be analyzed manually using exported Excel files or using Bio-Rad CFX Manager software. Here, we describe a comparative Ct method ($2^{-\Delta\Delta Ct}$) to analyze RT-PCR data. With the comparative Ct method it is possible to calculate the relative gene expression levels between different samples using the *Ct values* generated from RT-PCR [25].

Data Analysis Using Comparative Ct Method

Export all datasheets to Excel files.

1. Open the Excel file containing the *Ct* values. This analysis can be performed using Microsoft Office Excel or other statistical program.
2. Calculate the ΔCt for each target sample and for each reference sample. $\Delta Ct = Ct$ (target gene) $- Ct$ (reference gene). The target gene refers to your gene of interest and the reference gene refers to a standard housekeeping gene.
3. Calculate $\Delta\Delta Ct = \Delta Ct$ (target sample) $- \Delta Ct$ (reference sample). The result of this equation is the fold change of the target gene expression in the target sample, relative to a reference/control sample, normalized to the housekeeping genes (*see* **Note 17**) and do the graph (Fig. 2).

4 Notes

1. Use clean gloves while preparing all reagents and during RNA extraction, to avoid possible contaminants. Prepare purified deionized water, absolute ethanol, and 70% ethanol using purified deionized water. This is essential from the beginning to

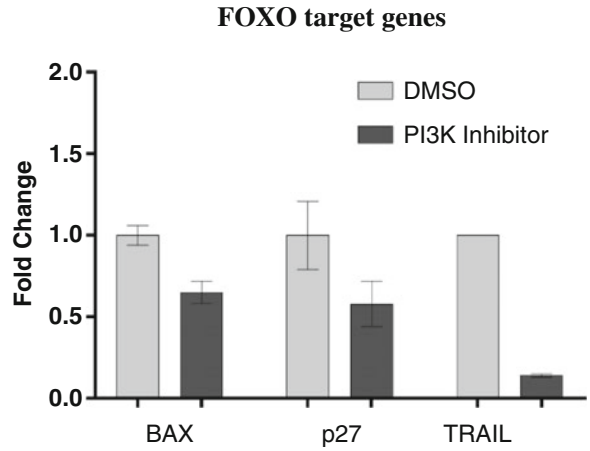


Fig. 2 Gene expression analysis of different FOXO target genes. Light grey bars represent U2OS cells treated with DMSO and dark grey bars represent U2OS cells treated with a PI3K inhibitor. mRNA expression levels were evaluated using RT-PCR and data was analyzed using Bio-Rad CFX manager 3.1 software. Y axis represents the fold change. GAPDH was used as a housekeeping gene

the end of work. RNA extraction is a critical step; RNA is quickly degraded by RNase, an enzyme present ubiquitously.

2. DNase treatment is an optional step that will remove any contaminant DNA present in your RNA samples. For this reason, we strongly advise to perform this step.
3. Pipette the 80 μL carefully, do not touch with the pipette tip on the column's membrane.
4. To avoid ethanol contamination on your RNA samples, **carefully** tap the collection tube on a filter paper sheet to remove theremaining wash buffer. Additionally, we also recommend an extra spin without adding any reagent, to guarantee that there is no ethanol in the sample.
5. Low 260/280 ratio usually indicates phenol contamination or low concentration of the nucleic acids. The Nanodrop2000 can also be contaminated from previous samples (e.g., Proteins). In this case we advise to repeat the procedure from **step 1**.
6. Include two negative controls: (1) one tube where no enzyme is added and (2) one tube where RNAse-free water is used instead of RNA. This set of controls will guarantee that there is no contamination with genomic DNA.
7. We recommend performing one Master Mix in order to reduce pipetting errors. Calculate the amount of Master Mix needed for all samples and then pipette the correct volume of Master Mix to each sample. Follwing, calculate the volume equivalent to 2 μg of RNA for each of your samples and subtract this amount to 20 μL – this value refers to the amount of water that you need to pipette to make up a total volume of 20 μL per reaction.

8. RNase H must be added in order to eliminate any remaining RNA following the reverse transcription reaction. The presence of remaining RNA may bind to cDNA and inhibit DNA synthesis. The inhibition of DNA replication might affect the Ct values.
9. It is recommended to dilute the cDNA at least five to tenfolds in order to avoid inhibitory effects on real-time PCR due the presence of non-cDNA contaminants (often carried out from impure RNA), or components of cDNA synthesis.
10. It is suggested to maintain the original cDNA stock and the diluted cDNA. If there is the need to run the samples again you use the same dilution and avoid thawing the original stocks unnecessarily.
11. The recommended volume for 96-well PCR plates is 15 μL and for the 384-well PCR plates is 10 μL. We recommend placing the PCR plate on a dark sheet of paperboard to increase the contrast and to help you keep track of the wells that have been loaded.
12. Prepare a scheme/layout of your PCR plate in Microsoft Office Excel discriminating the exact position of each sample as well as the primers needed for each one, as shown in Fig. 1. The scheme will help you while pipetting and designing the plate setup on the software latter.
13. We recommend making a master mix for each individual gene. To calculate the volumes needed for the pool master mix you have to consider the total number of reactions per gene (e.g., if you have five different samples and you perform three technical replicates and a non-template control, the total reactions per gene is 16). Add an 8% volume increment to each component of the master mix to account for pipetting errors. For a total volume of 10 μL reaction you must pipette: 0,40 μL of Primer Forward (at 10 μM concentration), 0,40 μL of Primer Reverse (at 10 μM concentration), 3,2 μL of DNase and RNase-free water, and 5 μL of Luminoct SYBR green and 1 μL cDNA (diluted 1:10). Considering the previous example, if you have 16 reactions per gene, you must prepare a master mix with 6.9 μL Primer Forward, 6.9 μL Primer Reverse, 55.3 μL water, 86.4 μL LuminoCt (8% error included), and then pipette 9 μL of this master mix per well, and pipette 1 μL cDNA per well. LuminoCt must be protected from light since it is light sensitive.
14. Carefully mix primers prior to use using a 200 μL pipette.
15. Use a new tip for each well that is being pipetted.
16. After sealing the PCR plate use a spatula to make sure that the plate is totally sealed and cut the excess.

Table 2
Representation of the comparative Ct method

	Target gene	Reference gene
Target sample—Treatment with drug	A	GAPDH 1
Reference /control sample	C	GAPDH 2

$\Delta Ct = Ct$ (target gene) − Ct (reference gene)
ΔCt (target sample) = Ct (A) − Ct (GAPDH 1)
ΔCt (Reference sample) = Ct (C) − Ct (GAPDH 2)
$\Delta\Delta Ct = \Delta Ct$ (target sample) − ΔCt (Reference sample)

17. Comparative *Ct* method is a useful method to calculate relative gene expression levels using *Ct* values generated by RT-PCR. Here we will present an example to help your data analysis. Imagine the following situation: analyze the expression of gene X before and after a treatment with drug A. In this case the target sample will be the sample after treatment, and the reference or control sample, the sample without treatment (Table 2). The housekeeping gene used was GAPDH.

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