Chapter 12

High-Throughput Real-Time PCR for Detection of Gene-Expression Levels

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Summary

While many high-throughput screening campaigns involve the measurement of protein levels or locations, at times it is desirable to measure the levels of gene expression in response to small molecules. Here, we describe a method for capturing mRNA in multiwell plates following compound treatment and measuring gene expression using real-time PCR. This streamlined protocol provides complementary information to conventional phenotypic cell-based assays, and is especially useful in cases where the gene of interest is thought to serve a regulatory function in downstream cellular phenotypes.

Key words: High-throughput gene expression, mRNA capture, Real-time PCR, Reverse transcription.

1. Introduction

High-throughput screening (HTS) involves the rapid assessment of large numbers of compounds for desired biological effects. Advances in technology and affordability have made feasible the use of HTS in an academic setting. Methods have been developed to measure, for example, protein expression *(1)*, protein binding *(2)*, and protein localization *(3)*. More recently, measurements of nucleic acids in response to small molecules have been developed. The evaluation of gene-expression levels is often used *after* HTS in order to identify the targets of small molecules of interest. However, there are circumstances in which the levels of gene expression themselves are the most relevant measurement for screening. In cases such as these, one can imagine two

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modalities for screening: first, the analysis of individual target-gene expression, which is especially appropriate for genes that are thought to serve regulatory functions, and second, the use of a multigene signature of a cellular phenotype, which works well for difficult-to-measure cellular outcomes. For the latter approach, the recent development of gene expression-based HTS (GE-HTS) *(4–6)* has enabled the simultaneous measurement of many transcripts, often in the context of identifying compounds that can induce cell-state switching, such as differentiation. This chapter will focus on the former screening paradigm, in which the analysis of individual target-gene expression is assessed in a high-throughput manner.

Overall, this method involves capturing mRNA from cells treated with compounds, and measuring gene expression using reverse transcription and real-time PCR (*see* **Note 1**). Cell lysis is performed within the compound-treated wells, and lysate is then transferred to a commercially available multiwell plate coated with oligo-dT for capture of mRNA. In-plate reverse transcription yields cDNA, which is in turn transferred to a PCR plate, where the gene of interest is amplified. Parallel measurement of a control gene allows normalization for mRNA content; this is particularly important for chemical screening, as compounds may nonspecifically affect cell number, viability, or per-cell mRNA content. This method results in the ability to assess the effects of a compound collection on individual gene expression.

2. Materials

2.1. Cell Culture

2.1.1. Plates

The choice of multiwell plates in which cells are cultured for screening is open to the discretion of the screener. In contrast to fluorescent and luminescence-based screening, the final step of detection will not be performed in the plates that are used for cell culture, so considerations such as clear bottoms or plate color are less important than in such cases. We have had success using Costar-brand clear cell-culture plates (Corning, Lowell, MA).

2.1.2. Culture Media

The protocol for capturing polyadenylated mRNA in multiwell plates can be used on adherent cells, suspension cells, or tissues. We have performed this protocol on adherent cells.

1. Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA).
2. Fetal bovine serum (FBS) to a final concentration of 5–10% (Invitrogen).
3. Penicillin-streptomycin solution (Invitrogen).

4. Phosphate buffered saline (PBS), pH 7.4.
5. Trypsin–EDTA solution (0.25%) (Invitrogen).

2.2. Capture of Polyadenylated mRNA

1. TurboCapture 384 mRNA kit (Qiagen, Valencia, CA). This kit includes the following materials: 384-well plates, the wells of which contain immobilized oligo-dT; buffer for in-well cell lysis; and wash buffer.
2. β-mercaptoethanol (commercial 14.3 M solution).

2.3. Amplification and Detection

1. iScript cDNA synthesis kit (BioRad, Hercules, CA).
2. Thermal cycler, such as the DNA Engine Tetrad (GMI, Ramsey, MN), capable of holding multiwell plates.
3. RNase-free water.
4. At least two high-throughput optical PCR plates (ABI, Foster City, CA), one or more for the gene(s) of interest, and one for a gene-expression control gene. This number of plates is needed for each compound stock plate; if screening multiple compound plates in replicate, the need for PCR plates obviously increases in a geometric fashion.
5. SYBR-green containing 2× PCR master mix (ABI).
6. Optical plate seals (ABI).
7. ABI Prism 7900, or similar real-time PCR intrument, using SYBR green as detector.

2.4. Robotics

2.4.1. Liquid Dispensing

1. In the absence of suitable liquid-handling robotics, a common laboratory multichannel pipettor will suffice for much of this protocol. However, the workload involved in screening will increase dramatically.
2. We use a μFill (BioTek, Winooski, VT) liquid dispenser for washing cells with PBS, as it is possible to adjust the dispense rate to levels slow enough to prevent disruption of the cell monolayers in each well.
3. For steps involving more precious reagents, we use a Multidrop Combi (Thermo Electron, Waltham, MA), favoring this instrument due to its very low dead volume and ability to recapture remaining reagent following dispensing.
4. The μFill dead volume is ~13 mL, while that of the Multidrop equipped with the low-volume manifold is only 1.5–2 mL.

2.4.2. Liquid Aspiration

1. As is the case with liquid dispensing, a multichannel aspirating wand (V & P Scientific, San Diego, CA) could suffice to remove media from multiwell plates.
2. However, we use an ELx405 plate washer (BioTek, Winooski, VT) to remove media in an automated fashion. This instrument

is capable of aspirating 96 wells in one motion, with Cartesian coordinates specified by the user.

2.4.3. Liquid Transfer

1. There are several steps in which the entire contents of a 384-well plate must be transferred to a new plate. While it may be feasible to imagine the use of a multichannel pipettor, there exist commercially available liquid-transfer robotics systems.
2. We use the CyBi-Well Vario (CyBio, Boston, MA), which can be equipped with tip trays containing 96 or 384 pipet tips, capable of transferring up to 250 or 25 μL each, respectively.

3. Methods

3.1. Cell Culture

1. Seed cells in multiwell plates. Again, the choice of plates used for this step is open to the user. To ensure that we had sufficient mRNA for detection, we used Costar brand 96-well plates containing a confluent monolayer of primary mouse muscle cells.
2. Thus, four 96-well plates of cells are needed for each 384-well compound plate.
3. In general, 10,000 cells per well of a 96-well plate results in a confluent layer; for mouse models of muscle that require differentiation, such as C2C12, we seed 8,000 per well and start differentiation the following day by replacing with media containing 2% horse serum (*see* **Note 2**).
4. Such cells require 4–6 days for differentiation, but the primary muscle cells, which have been isolated and frozen in liquid nitrogen, express markers of differentiation in 1 day.
5. Allow cells to adhere to wells. While many cell lines will adhere to the cell culture-treated multiwell plate in several hours, we allow the cells to incubate at least overnight in a water-jacketed cell-culture incubator containing 5% CO_2 and at 95% humidity.

3.2. Compound Treatment

1. Replace plates with fresh media. The pH and nutrient levels of the cell-culture media do not remain stable in multiwell plates for more than roughly 2 or 3 days, so we typically replace the plates with 100 μL/well fresh media immediately before adding compounds.
2. Add compounds. A more detailed discussion of compound sources is addressed elsewhere *(7, 8)*. There exist several technologies for compound treatment, including pin-transfer *(9)*, acoustic transfer *(10)*, or small-molecule microarrays *(11)*.

3. Using the first method listed in **step 2**, we used a CyBi-Well (CyBio) equipped with a 96-pin array to add 100-nL compound to each well of the 96-well plate of cells. The steel pins are dipped into a compound plate, and subsequently dipped into the cell-culture media, allowing the dispersal of compound (*see* **Note 3**).
4. Washing the pin array in DMSO (the solvent in which the compounds are dissolved) and methanol allows this process to be repeated.
5. This process results in a 1,000-fold dilution of compound, typically yielding screening concentrations of 10–20 μM.
6. We incubated the cells with compounds for 24 h, though the amount of treatment time depends on the immediacy of induction expected for the gene of interest.
7. To maximize gas exchange between stacks of multiwell plates, we used specially designed plate carriers containing shelf-like slots for each plate (please contact the corresponding author for specifications).

3.3. Preparing mRNA

1. Wash cells once with PBS, using either 100 μL/well (for a 96-well plate) or 50 μL/well (for a 384-well plate). When using a liquid-dispensing robot, be sure to employ a low dispense rate to prevent cells from becoming dislodged from the bottom of the well.
2. Aspirate wells fully, ensuring that no liquid remains in the well. Manual aspiration works well for this step, as do several other techniques (*see* **Note 4**).
3. Add β-mercaptoethanol to the commercial lysis buffer to a final concentration of 1% (v/v), and add 50-μL lysis buffer to each well.
4. Incubate plates for 5 min at room temperature. No shaking is required at this step (*see* **Note 5**).
5. Transfer 30 μL of the lysate to the TurboCapture plate. As mentioned earlier, it is technically possible to use a multichannel pipettor for this transfer step. However, an automated liquid handler speeds up the process considerably (*see* **Note 6**). We used a CyBi-Well Vario (CyBio) equipped with disposable 96-tip arrays, thus requiring four tip trays for each 384-well TurboCapture plate. We have found that transferring 30 μL of the 50-μL lysate yields sufficient transcript detection, and prevents any problems resulting from the pipet tips hitting the bottom of the cell-culture plate.
6. Incubate for 60–90 min with shaking at 100 rpm (*see* **Notes 7** and **8**). Any flat orbital shaker will suffice.
7. Wash plates three times with the proprietary wash buffer included with the TurboCapture kit, making sure that the last step is aspirated fully (*see* **Note 4**).

3.4. RT-PCR

1. To the now-empty TurboCapture plate, add 5 µL/well iScript cDNA synthesis mix. The use of random primers (rather than oligo-dT) is favored here, since the mRNA poly(A) tails are already annealed to the oligo-dT on the plates. The use of no primers is not a good option, as it will result in cDNA that is covalently bound to the plate.
2. Program thermal cycler, and allow reverse transcription to proceed. We used the scheme recommended by the manufacturer: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C (*see* **Note 9**).
3. Add 20 µL/well RNase-free water, using a liquid dispenser such as those listed in "Materials." This step results in 25-µL total cDNA solution (*see* **Note 10**).
4. For each TurboCapture plate, prepare two PCR plates: one for your transcript of interest, and one for the control transcript (e.g., TATA box-binding protein, or TBP). To each well, add 3-µL master mix (2.5 µL of 2× master mix, plus 0.25 µL of 1 µM each DNA primer to be used for PCR amplification). We have used standard two-probe quantitative PCR, with free SYBR in the mix, with good outcomes. In principle, TaqMan probes could be used at this point.
5. Add 2 µL/well cDNA to each PCR plate. The CyBi-Well Vario, equipped with a 384-pipet tip tray, can attain this precision, and we found this instrument very well suited for this step, as cDNA could be pipetted iteratively into multiple PCR plates, depending on the number of transcripts to be evaluated (*see* **Note 11**).
6. Perform PCR, using an automated instrument such as listed in "Materials." We have used the standard ΔΔCt method for calculating gene expression (*see* **Note 12**), roughly as follows. An arbitrary threshold is chosen, and the cycle number at which signal for the gene of interest crosses that threshold in each well is recorded. This value is then subtracted from the value acquired when measuring the TBP control. That value, in turn, is subtracted from the average value similarly acquired from control wells (typically a few dozen). The output represents the log (base 2) of fold-induction of gene expression.

4. Notes

1. Before commencing with full-scale small-molecule screening, we suggest careful assay development. For example, one should determine the coefficient of variation (CV) within the data. This can be determined by dividing the arithmetic mean

of the data by the standard deviation – in this case, normalized cycle number. We measured gene expression on an entire 384-well plate, half of which was treated with 0.1% DMSO, corresponding to the final concentration of solvent after compounds are pin-transferred into these plates, and half of which contained a positive control for increased expression of the gene of interest.

2. We have tested this protocol with other cell types, and noted that primary mouse adipocytes, upon lysis, result in a mixture that is too viscous for reliable pipetting using our liquid transfer systems. We suggest performing a small-scale test lysis on the cells of interest before proceeding with large-scale assay development.
3. During the course of any HTS campaign, it is important to keep compound and cell-culture plates in the same orientation, so as to ensure that well A01, for example, truly receives the compound residing in well A01 of the compound plate. In this protocol, we have described several steps requiring transfer of materials: compound addition to the cell-culture plate, consolidation from 96-well format to 384-well format, and transfer of cDNA from TurboCapture to PCR plates. Thus, it is vitally important to maintain orientation uniformity throughout the protocol, either by careful manual labeling or by the use of, for example, barcoding and scanning techniques.
4. There are several steps in this protocol that call for "full aspiration" of the liquid in each well. We have observed that automated plate washers tend to leave a very small but nonzero amount of liquid in each well (1–3 μL/well). A few simple methods are effective at removing this last volume. First, the plate may be centrifuged upside-down. In this case, the plate is placed in a centrifuge plate carrier, on top of several paper towels, and spun for 1 min at 1,000 rpm. This method is the surest way of removing all liquid from a plate, and is sufficiently gentle that disruption of the immobilized mRNA is prevented. Second, and a perhaps even more low-technology option, is that one may literally shake the remaining liquid volume out of the plate; this involves turning the plate upside down over a biohazard bag and applying a rapid downward snap.
5. The addition of lysis buffer to the cell-culture plates will cause lysis to occur nearly instantaneously. The user can verify this lysis by microscopic examination. There is no need for shaking. The 5-min incubation is indicated in order to ensure full lysis, and appears to be a conservative estimate.
6. When deciding between using a multichannel pipettor and liquid-dispensing robotics, it is useful to consider the number of plates to be processed, and the dead volume required. For example, precious reagents such as the PCR mix, at 3 μL/well,

require 1.15 mL per 384-well plate. The dead volume for the Multidrop Combi is 1.5–2 mL, more than 100% of what is needed for a single plate. However, if ten plates are being prepared at a sitting, 11.5-mL PCR mix is required, and the dead volume is now 13–18% of the total volume, making the automated dispensing more attractive and economical.

7. Once lysate is added to the TurboCapture plates, they should be centrifuged to assure that the lysate is in contact with the bottom of the well. One minute at 1,000 rpm is sufficient for this purpose.
8. The TurboCapture plate containing cell lysate should be incubated with shaking for at least 60 min at room temperature, but we have not observed better assay results by increasing that length of time.
9. For the reverse transcription step, a multiplate thermal cycler is recommended, as there is a 40-min interval for each plate. Thus, the ability to parallelize this process will result in the ability to process more plates per day.
10. Following the addition of RNase-free water, there is no need to transfer the cDNA to a new plate before distributing aliquots to PCR plates. The reduction in temperature from 85°C to room temperature will *not* cause the cDNA to reanneal to the TurboCapture well surface.
11. If the choice is made to use a multichannel pipettor when distributing aliquots of cDNA, it is important to remember that the tips must be changed for every change of row or column. Otherwise, well-to-well contamination will occur. The use of an automated liquid-transfer robot is ideal, but this is an important consideration if using more manual methods.
12. We have found the reproducibility of this assay to be very high. Under our conditions, the standard deviation for the ΔΔCt value was less than 0.5 (i.e., 1.4-fold induction). We have found that performing PCR once using the control gene, such as *TBP*, is sufficient for subsequent PCR runs. Thus, with 25-μL total cDNA yield, and at 2-μL cDNA per PCR experiment, one could theoretically measure the expression levels of 12 genes, including the control gene. In reality, pipetting error and cost considerations result in this number being typically lower.

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