Chapter 2

Global RT-PCR and RT-qPCR Analysis of the mRNA Expression of the Human PTPome

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Abstract

Comprehensive comparative gene expression analysis of the tyrosine phosphatase superfamily members (PTPome) under cell- or tissue-specific growth conditions may help to define their individual and specific role in physiology and disease. Semi-quantitative and quantitative PCR are commonly used methods to analyze and measure gene expression. Here, we describe technical aspects of PTPome mRNA expression analysis by semi-quantitative RT-PCR and quantitative RT-PCR (RT-qPCR). We provide a protocol for each method consisting in reverse transcription followed by PCR using a global platform of specific PTP primers. The chapter includes aspects from primer validation to the setup of the PTPome RT-qPCR platform. Examples are given of PTP-profiling gene expression analysis using a human breast cancer cell line upon long-term or short-term treatment with cell signaling-activation agents.

Key words Protein tyrosine phosphatase, Reverse transcription PCR, Real-time quantitative PCR, PTPome

1 Introduction

The global analysis of changes in the expression of well-defined gene or protein families during physiological or pathological conditions provides valuable information to understand the regulation of cell physiology in health and disease. Genome-wide DNA microarray analyses generate reliable comparative global information on gene expression patterns and on major changes in the expression of individual genes. However, the wide scope of genome-wide analysis may hamper the optimization and comparison of the gene expression changes on specific groups of genes. In addition, small changes in gene expression, especially for genes expressed at low levels, may be overlooked due to the limited sensitivity of the DNA microarray assays [1–3]. Reverse transcription quantitative real-time PCR (RT-qPCR) may overcome these problems because of its very high sensitivity and dynamic range and the possibility of individualized choosing and optimization of primers specific for
the genes of interest, including mRNA isoform-specific primers [4, 5]. Semi-quantitative reverse transcription PCR (RT-PCR) provides limited quantitative information, but can be useful to compare the expression of related mRNA variants which can be differentiated by size, or the relative expression of a mRNA of interest versus a reference mRNA, if coupled to agarose gel electrophoresis [6, 7].

The protein tyrosine phosphatase (PTP) superfamily is composed of members that belong to several gene families, which differ in the number and type of catalytic domains, as well as in their catalytic mechanism and overall substrate specificity. As such, an array of enzymes or enzyme-like proteins directly related with Tyr dephosphorylation can be considered as the PTPome. A former version of the human PTPome was defined as formed by about 107 members, and in an extended updated form expands to about 125 members [8, 9]. Many PTPome members harbor different noncatalytic regulatory domains, whereas others are small proteins only composed of the catalytic domain [8, 10–18]. The classical PTP enzymes (class I Cys-based classical PTPs) and the dual-specificity (DSPs) VH1-like PTPs (class I Cys-based DSPs) form the core of the PTPome and account for most of its members. They harbor one or two (classical PTPs), or only one (DUSPs), conserved catalytic PTP domains, and contain the conserved HCxxGxxR catalytic signature motif. The classical PTPs form a homogeneous group of enzymes with substrate specificity mostly restricted towards pTyr residues, whereas the DSPs include several subfamilies with different substrate specificities, including specificity towards pTyr/pSer/pThr, phosphoinositides, phosphorylated carbohydrates, and mRNA. Other PTPome enzymes contain a less-conserved CxxxxxR catalytic motif and employ a Cys-based catalysis to dephosphorylate pTyr/pSer/pThr residues or phosphoinositides, but harbor different classes of catalytic domains [arsenate reductase domain (class II-Cys based); rhodanese domain (class III Cys based)]. Finally, some phosphatases dephosphorylate pTyr using catalytic mechanisms non-Cys based, which belong to independent phosphatase gene families (Asp-based phosphatases, His-based phosphatases) [9]. This is of importance since the catalytic active sites of each of these enzyme families, as well as the physiologic regulation of their activities, display differences, making possible phosphatase family specific drug targeting with experimental or therapeutic purposes.

The PTPome is well suited to perform RT-PCR and RT-qPCR approaches to analyze comparatively the gene expression of their members [19–23]. We describe here methods to perform comparative semi-quantitative (RT-PCR) and quantitative (RT-qPCR) analysis of the gene expression of the human PTPome. Examples are shown using the MCF-7 human breast cancer cell line grown under different experimental conditions.
2 Materials

All solutions are prepared in double-distilled, RNase-free water. Cell culture and transfection procedures require sterile conditions.

1. Tissue-cultured cells, or biological samples, as a source of RNA.
2. Specific primer sets for amplification of the PTPs of interest and the reference control genes (see Notes 1 and 2).
3. RNA isolation kit (see Note 3).
4. cDNAs of the PTPs of interest, for primer specificity validation.
5. RT and PCR reagents (see Note 4).
6. Thermocycler.
7. Agarose gel electrophoresis and DNA visualization reagents (see Note 5).
8. Ultraviolet light detection system.

2.1 Analysis of Human PTPome mRNA Expression by Semi-quantitative RT-PCR

1. Tissue-cultured cells, or biological samples, as a source of RNA.
2. Validated primer sets for quantitative RT-PCR (RT-qPCR) (see Note 6).
3. RT-qPCR reagents (see Notes 4 and 7).
4. RNA isolation kit (see Note 3).
5. cDNAs of the PTPs of interest, for primer amplification efficiency validation.
6. Plate setup suitable for loading the qPCR PTPome set.
7. Real-time thermocycler.

2.2 Global Analysis of Human PTPome mRNA Expression by Quantitative RT-PCR

The first method (Subheading 3.1) is aimed to monitor semi-quantitatively (band intensity) and qualitatively (band size) the expression of mRNAs from different related PTPs under different experimental conditions. This approach is useful when the relative size of the amplified bands provides information on the expression of highly related PTP isoforms or variants, which may display different functional properties [7, 24]. In addition, this methodology can provide a good and sensitive overall view of the relative expression of the members of PTP subfamilies (see Fig. 1). The second method (Subheading 3.2) is designed to perform a global and quantitative monitoring of the mRNA expression of the PTPome under different experimental conditions. Our setting uses real-time RT-qPCR methodology scaled to accommodate, in 96-well (96-w) or in 384-well (384-w) plates, primers that amplify individually the members of the PTPome in a single experiment (see Fig. 3).
1. Design specific oligonucleotide primer sets for the group of PTPs of interest, and perform PCR tests for primer specificity and cross-reactivity using as templates plasmids containing the distinct PTP cDNAs (see Note 8). An example of primer specificity test, using primers that amplify the members of the human MKP family of active PTPs (Table 1), is shown in Fig. 1a. Each PCR reaction contained 10 ng template, 0.5 mM dNTP mix (2.5 μl from 10 mM stock [2.5 mM each dNTP]), 0.3 μM of each primer (1.5 μl from 10 μM stock),
and 1 U GC-rich DNA Polymerase (0.5 µl from 2 U/µl stock) (see Note 4) in a final volume of 50 µl. PCR conditions were a denaturation step, 95 °C, 5 min, followed by 35 cycles: denaturation, 95 °C, 1 min; annealing, 55 °C, 2 min; extension, 72 °C, 1 min.

2. Isolate total RNA, or poly(A) RNA, from the cells or tissues of interest (see Note 3) and measure RNA concentration and purity in a nanodrop spectrophotometer (see Note 9).

3. Incubate (12.5 µl final volume) 1 µg total RNA, or 10 ng poly(A) RNA (see Note 3), and oligo(dT)18 primers (1 µl from 100 µM stock) at 70 °C, 10 min to denature RNA secondary structure, and transfer to ice to let the primers to anneal to the RNA.

Table 1

<table>
<thead>
<tr>
<th>Gene/protein</th>
<th>Sense primer (5′–3′)</th>
<th>Antisense primer (5′–3′)</th>
<th>Amplified fragment size (bp)</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>DUSP1/MKP1</td>
<td>GTG GGC ACC CTG GAC GCT</td>
<td>GCT GAG CCC CAT GGG GGT</td>
<td>426</td>
<td>C-term</td>
</tr>
<tr>
<td>DUSP4/MKP2</td>
<td>GAC TGC AGT GTG CTC AAA AGG</td>
<td>AAC Cgg GGG TGG CAT GGC</td>
<td>483</td>
<td>C-term</td>
</tr>
<tr>
<td>DUSP6/MKP3</td>
<td>ATA GAT ACG CTC AGA CCC GTG</td>
<td>CTC GCC GCC GTT ATT CTC G</td>
<td>333</td>
<td>C-term</td>
</tr>
<tr>
<td>DUSP9/MKP4</td>
<td>GAG GGT CTG GGC CGC TCG</td>
<td>CGC CAT GCT GGA GCC GGC</td>
<td>450</td>
<td>C-term</td>
</tr>
<tr>
<td>DUSP10/MKP5</td>
<td>GCA CTA TCT AGG CCC GTG C</td>
<td>Gtt GTA CTC CAT GAA GGG CC</td>
<td>516</td>
<td>C-term</td>
</tr>
<tr>
<td>DUSP16/MKP7</td>
<td>CAT GAG ATG ATT GGA ACT CAA A</td>
<td>AGG GAC TAG AGT GGA TTT TCC T</td>
<td>426</td>
<td>C-term</td>
</tr>
<tr>
<td>DUSP5</td>
<td>TCG CTC GAC GGG CGC CAG</td>
<td>CTC ACT CTC AAT CTT CTC TTG T</td>
<td>450</td>
<td>C-term</td>
</tr>
<tr>
<td>DUSP2</td>
<td>CTG GAG TGC GCG GGG CTG</td>
<td>CAG CGC AGG GGC GGG G</td>
<td>429</td>
<td>C-term</td>
</tr>
<tr>
<td>DUSP8</td>
<td>GGG GAC CGG CTC CGG AG</td>
<td>GCT CAT GGG TAG CAG GGC A</td>
<td>438</td>
<td>C-term</td>
</tr>
<tr>
<td>DUSP7/MKPX</td>
<td>AAC GCC TTC GAG CAC GGC G</td>
<td>GGA CTC CAG CGT ATT GAG TG</td>
<td>408</td>
<td>N-term</td>
</tr>
<tr>
<td>ACTB/β-actin</td>
<td>CCA AGG CCA ACC GCG AGA AGA TGA C</td>
<td>AGG GTA CAT GGT GGT GCC GCC AGA C</td>
<td>562</td>
<td>Core</td>
</tr>
</tbody>
</table>

*Primers were used for experiments shown in Fig. 1

*The protein region targeted by each pair of primers is indicated
4. To perform the reverse transcription reaction, add 4 mM dNTP mix (2 μl from 40 mM stock [10 mM each dNTP]), RNase inhibitor (0.5 μl, 20 U), reverse transcriptase (RT; 1 μl from 200 U/μl stock), and RT buffer (4 μl 5×), to give a final volume of 20 μl (see Note 4). Incubate at 42 °C, 1 h.

5. Optional: Incubate at 70 °C, 10 min, to inactivate the RT enzyme.

6. Measure concentration and purity of cDNA in a nanodrop spectrophotometer (see Note 10).

7. Perform the PCR reaction using the validated primer sets and 50–200 ng cDNA/reaction, as described in step 1 (see Note 11).

An example of the relative expression of the mRNAs from the MKP family of PTPs, in human breast carcinoma MCF-7 cells untreated or treated with phorbol 12-myristate 13-acetate (PMA), is shown in Fig. 1b.

The high sensitivity of RT-qPCR makes necessary maximal accuracy in the manipulation and processing of the samples, as well as in the design and control of the experiment itself [25].

1. Choose or design the appropriate set of oligonucleotide primers sets for the members of the PTPome and for the reference genes (see Note 6). It is recommended to test individually the specificity of the primers by checking that a single sharp peak is obtained in the melt curve at the end of the PCR reaction (see Note 12).

2. Efficiency of the primers: it is recommended (especially when the primers are not prevalidated commercially) to test that the efficiency (E) of the amplification reaction is close to 2.0 (two-fold amplification per cycle = 100% efficiency). This can be calculated from the slope of the quantification cycle (Cq) standard curve, which is made by running PCR reactions using decimal dilutions (e.g. 1:1, 1:10, 1:100) of the cDNA to be analyzed or of a template plasmid (starting with 10 ng) containing the cDNA amplified by the primers, and representing Cq (y) vs. LOG template concentration (x) \[ y = \text{slope} \times x + C_q (x=0); \ E = 10^{(-1/\text{slope})} \] (see Note 13). The Pearson’s correlation coefficient (r) of the standard curve should be >0.990. Examples of Cq standard curves for a set of commercial primers (QIAGEN) for the human MKP family of active PTPs, and the calculation of the efficiency for one of them (DUSP10), are given in Fig. 2.

3. Obtain cDNA by retrotranscription of the RNA of interest, as in Subheading 3.1, to be used as template.

4. Setup of plates: primers for all PTPs and controls are aliquoted at 10 μM in stock plates (Fig. 3). From the stock plate, a multipipette is used to make 384-w PCR working plates
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**Fig. 2** Validation of qPCR primers for efficiency. (a) Primer efficiency \( E \) \( E = 10^{-\frac{1}{Slope}} \) of human MKP primers is calculated from the slope of the plot, generated by running reactions using decimal dilutions (1:1, 1:10, and 1:100) of a template cDNA (starting at 100 ng) from control cells. (b) An example for DUSP10 primers is shown in the bottom.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Dilutions</th>
<th>times</th>
<th>LOG</th>
<th>Cq</th>
<th>Slope</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>DUSP10</td>
<td>1:1</td>
<td>100</td>
<td>2</td>
<td>26.95</td>
<td>-3.16</td>
<td>2,069930511</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>10</td>
<td>1</td>
<td>30.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>1</td>
<td>0</td>
<td>33.28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 3** Setup for the RT-qPCR analysis of the PTPome. A stock plate with primer aliquots for each PTP (numbered as 1, 2, etc., as an example) is used to transfer the primers to the PCR working plate, in triplicate (1.1, 1.2, 1.3, 2.1, 2.2, 2.3, etc., as an example) or in duplicate (not shown), for both untreated and treated conditions. Reaction mix is then added to the working plate and PCR is run.
containing 2 μl of each primer set per well, in duplicate or in triplicate, including in the same plate the experimental conditions to be compared (e.g., “untreated” vs. “treated”) (Fig. 3) (see Note 14). All plates are always kept on ice.

5. Reaction mix (20 μl/reaction): 2 μl primers, 5 μl cDNA (50–200 ng) in PCR-grade H₂O, 3 μl PCR-grade H₂O, 10 μl Master Mix (2×) (containing DNA polymerase, dNTPs, Dye, and reaction buffer) (see Note 7).

6. Add reaction mix with a multipipette. When pipetting of working plate is finished, cover the plate with sealing foil and keep covered with aluminum foil, on ice, until placed in the thermocycler.

7. Run the reaction (see Note 15). An example of the mRNA expression of most of PTPome members from MCF-7 cells untreated or treated with PMA for 72 h, using a set of commercial primers (QuantiTect, QIAGEN), is shown in Fig. 4. PCR conditions were a denaturation step, 95 °C, 10 min, followed by 40 cycles: denaturation, 95 °C, 15 s; annealing, 55 °C, 20 s; extension, 72 °C, 15 s.

8. Analyze the data using the appropriate software. A common way to present the data is in logarithmic scale (LOG₂), where significant changes are usually considered > or equal to 2, or < or equal to −2. For relative changes, fold change can be calculated using the ΔΔCq equation: ΔΔCq = 2\((Cq_{untreat} - Cq_{treat}) - (Cq_{untreat} - Cq_{untreat})\); where the Cq from the reference (ref) genes is subtracted to the Cq of each PTP in both the “untreated” (untreat.) and “treated” (treat.) conditions. For the choosing of reference genes, see Note 16.

Fig. 4 Example of RT-qPCR expression analysis of the PTPome in MCF-7 cells treated with PMA. MCF-7 cells were left untreated or treated with PMA (50 ng/ml) for 72 h, and total RNA was purified and used for retrotranscription. cDNA was processed for PCR using a set of commercial primers (QuantiTect, QIAGEN). For this example, PCR was performed using technical duplicates. Relative expression values in LOG₂ scale are shown. Only a fraction of the PTPome, ordered from lower to higher fold change, is shown.
As an example, we show the mRNA expression of MKPs from MCF-7 cells stimulated with Epidermal Growth Factor (EGF), an ERK1/2-activating stimulus (Fig. 5). Six MKPs showed significant changes in gene expression after 24 h of EGF treatment, which declined after 48 and 72 h. The highest upregulated MKPs were DUSP4, DUSP6, and DUSP5 (Fig. 5a). In Fig. 5b, a comparative schematic kinetics of ERK1/2 activation, and DUSP4, DUSP6, and DUSP5 mRNA upregulation by EGF, is shown.

![Graph showing mRNA expression of MKPs](image)

**Fig. 5** (a) RT-qPCR analysis of the mRNA expression of the human MKP family of active PTPs from MCF-7 cells treated with EGF. MCF-7 cells were left untreated or treated with EGF (50 ng/ml) 24, 48, and 72 h, and processed for RT-qPCR as in Fig. 4. Mean fold change in gene expression is represented as LOG2. DUSP4, DUSP6, and DUSP5 were significantly upregulated. (b) Example of coordination of ERK1/2 activation and DUSP4, DUSP6, and DUSP5 expression during transient activation of ERK1/2 by EGF stimulation. Data are normalized from (a) and from [21].

As an example, we show the mRNA expression of MKPs from MCF-7 cells stimulated with Epidermal Growth Factor (EGF), an ERK1/2-activating stimulus (Fig. 5). Six MKPs showed significant changes in gene expression after 24 h of EGF treatment, which declined after 48 and 72 h. The highest upregulated MKPs were DUSP4, DUSP6, and DUSP5 (Fig. 5a). In Fig. 5b, a comparative schematic kinetics of ERK1/2 activation, and DUSP4, DUSP6, and DUSP5 mRNA upregulation by EGF, is shown.

4 Notes

1. When comparison between groups of PTPome members is going to be done, the primers should target nonconserved regions, and amplify fragments of similar size (see also Notes 8 and 12).
2. The reference gene chosen for the experiment shown in Fig. 1b is ACTB/β-actin. Note that the expression of ACTB/β-actin mRNA, as that of many other commonly used reference genes, may change under your study conditions [26, 27]. An evaluation of the more appropriate reference genes for each study conditions should be made [28, 29].

3. There are many kits suitable for isolation of total or poly(A) RNA. As a rough estimation, poly(A) RNA constitutes 1% of total RNA cellular content, which has to be taken in consideration for the amount of RNA used in the RT reaction. In general, total RNA works well for a reliable relative quantification of large sets of different target mRNAs [30]. For the experiments shown here, we have used Illustra QuickPrep Micro mRNA Purification Kit and IllustraRNAspin Mini Kit (GE Healthcare Life Sciences).

4. Many retrotranscriptase and DNA-polymerase options are available. For analytical purposes (nonpreparative), any high-amplification efficiency and high GC content tolerant Taq polymerase is well suited. We commonly use RevertAid™ reverse transcriptase, oligo(dT)18 primers (stock 100 μM), and Ribolock RNase inhibitor from Fermentas. In the experiments shown in Fig. 1, GC-rich DNA polymerase from Roche was used. dNTPs mixes often come in the Master mix of some commercial kits or can be purchased separately. For RT, we use a commercial dNTP mix (4×10 mM; 40 mM total) from Fermentas. For PCR, we prepare a stock dNTP mix 4×2.5 mM (10 mM total) from the individual dNTPs.

5. When small differences in size (for amplicons between 250 and 750 bp), or when small amplicons have to be compared, 1%–2% agarose gels are convenient. The gels shown in Fig. 1 were visualized by staining with 0.6 μg/ml ethidium bromide, but alternative less-toxic DNA stainers, such as GelRed™ or GelGreen™, are already available.

6. There are a wide variety of programs and online tools for qPCR primer design (see, for instance, http://molbiol-tools.ca/PCR.htm). When a relatively large number of different genes are going to be analyzed, as in the case of the PTPome analysis, the use of sets of prevalidated commercial primers from the same source gives the advantage of more homogeneous optimal amplification conditions. In the experiments shown in Figs. 2, 4 and 5, we have used QuantiTect Primer Assays (QIAGEN) primers.

7. In the experiments shown here, we have used SYBR® Green-based reagents (Roche Applied Science), but other dyes are also suitable for use.

8. For proper comparison, the DNA amplified fragments should be of similar size, and the pairs of primers should have a similar
Tm. For the experiments shown in Fig. 1, the length of the MKP primers was between 18 and 22 mer, and the Tm (calculated as \((G+C) \times 4 + (A+T) \times 2\)) was 62–64.

9. The ratio of absorbance at 260/280 nm for RNA should be around 2.0.

10. The ratio of absorbance at 260/280 nm for cDNA should be around 1.8. Sometimes removal of the template RNA is necessary by treating the RT reaction with RNase H before performing the PCR reaction.

11. In this method, the amplified band is the end product of the PCR reaction. This makes important to work with amounts of RNA and cDNA that allow visualization of differences. The number of PCR cycles for semi-quantitative PCR should be optimized to avoid oversaturation of the PCR reaction product. 30–35 cycles is a good range depending on the relative intensity of the bands of interest. To avoid false negatives and false positives, sometimes it is convenient to separate the reactions in two groups and amplify each group with different number of cycles. Note that in such case, comparisons have to be made within each group of PTPs.

12. A typical run for the melting curve is 95 °C, 15 s; 55 °C, 40 s. An additional control of specificity is to run the PCR product on an agarose gel to check the appropriate size of the amplicon. Note that sometimes amplicons from qPCR are of small size (see Note 5).

13. In practical terms, for a standard curve made with decimal dilutions of template, \(E=2\) means that 3.32 cycles more are needed to reach the Cq when using ten times less template (slope = −3.32 means 100% efficiency).

14. It is convenient to have the PTPome primers in a 384-w primer stock plate, and make the transferring to the PCR working plate using a 12-channel 384-w multipipette. Alternatively, transferring can be made using an 8-channel 96-w multipipette, but taking six alternate wells each transfer, two transfers per row (an 8-channel 96-w multipipette will dispense samples to alternate wells in a 384-w plate). For two conditions (“untreated” vs. “treated”) and technical triplicates, 48 genes can be analyzed in one 384-w plate. For two conditions (“treated” vs. “untreated”) and technical duplicates, 72 genes can be analyzed in one 384-w plate. It is recommended to use technical triplicates.

15. There are different qPCR devices and detection systems, which have to be compatible. In the experiments shown here, we have used a LightCycler™ 480 thermocycler and the corresponding SYBR™ Green I Master Mix (Roche Applied Science).
16. The reference genes chosen for the experiments shown in Figs. 4 and 5 were HPRT1/hypoxanthine phosphoribosyltransferase 1, ACTB/β-actin, and GAPDH/glyceraldehyde-3-phosphate dehydrogenase (see also Note 2).

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