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Adapter-tagged competitive PCR (ATAC-PCR) – a high-throughput quantitative PCR method for microarray validation

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Abstract

Adapter-tagged competitive PCR (ATAC-PCR) is an advanced version of competitive quantitative PCR that is characterized by the addition of unique adapters to cDNA derived from each sample RNA. Using multiple adapters, we can accurately measure the relative expression ratios of many samples, with a calibration curve obtained from internal standards included in the same reaction. ATAC-PCR can identify differences in gene expression as small as twofold, even from very small amounts of sample RNA. This technique is suitable for confirming results obtained with cDNA microarrays or differential display, and it can process more than a thousand of genes per day when used in conjunction with a capillary DNA sequencer.

Keywords: Adapter-tagged competitive PCR; Gene expression profiling; Laser capture microdissection

1. Introduction

One of the most prominent trends in molecular biology today is the increasing emphasis on understanding biological processes in terms of structures and the interactions of individual molecules. While untangling and understanding the complex control networks of gene expression and cascades of gene function present formidable challenges, gene expression profiling is a powerful and effective approach in addressing these issues. Large-scale quantification of intracellular transcript levels currently relies on hybridization-based and sequence-based expression analysis, including DNA microarrays [1], DNA chips [2], serial analysis of gene expression (SAGE) [3], and digital expression profiling [4]. Yet none of these methods are capable of accurately estimating small differences in expression levels among low-abundance transcripts [5].

We introduced the ''adapter-tagged competitive PCR (ATAC-PCR)'' method in order to make more precise measurements of gene expression ratios between RNA samples [6]. Since this technique does not require the time-consuming experimental construction of calibration curves, several hundred genes can be assayed simultaneously. We have previously used ATAC-PCR to analyze gene expression in the developing mouse brain [7–10] and in cultured neural cells [11], thereby demonstrating its potential applications in neuroscience. The technique is capable of not only identifying differences in gene expression as small as twofold, but also analyzing very small amounts of RNA; thus, it can be used with RNA isolated by laser capture microdissection (LCM) and other techniques with small yield. These features make ATAC-PCR an ideal method for the confirmation of candidates identified by microarray experiments. In genomic research laboratories, ATAC-PCR can even be used for gene expression profiling, instead of DNA microarrays.

2. Outline of the ATAC-PCR method

ATAC-PCR is an advanced form of quantitative PCR, which is characterized by the ligation of different adapters to cDNA derived from each sample RNA. A schematic diagram of the ATAC-PCR method is shown

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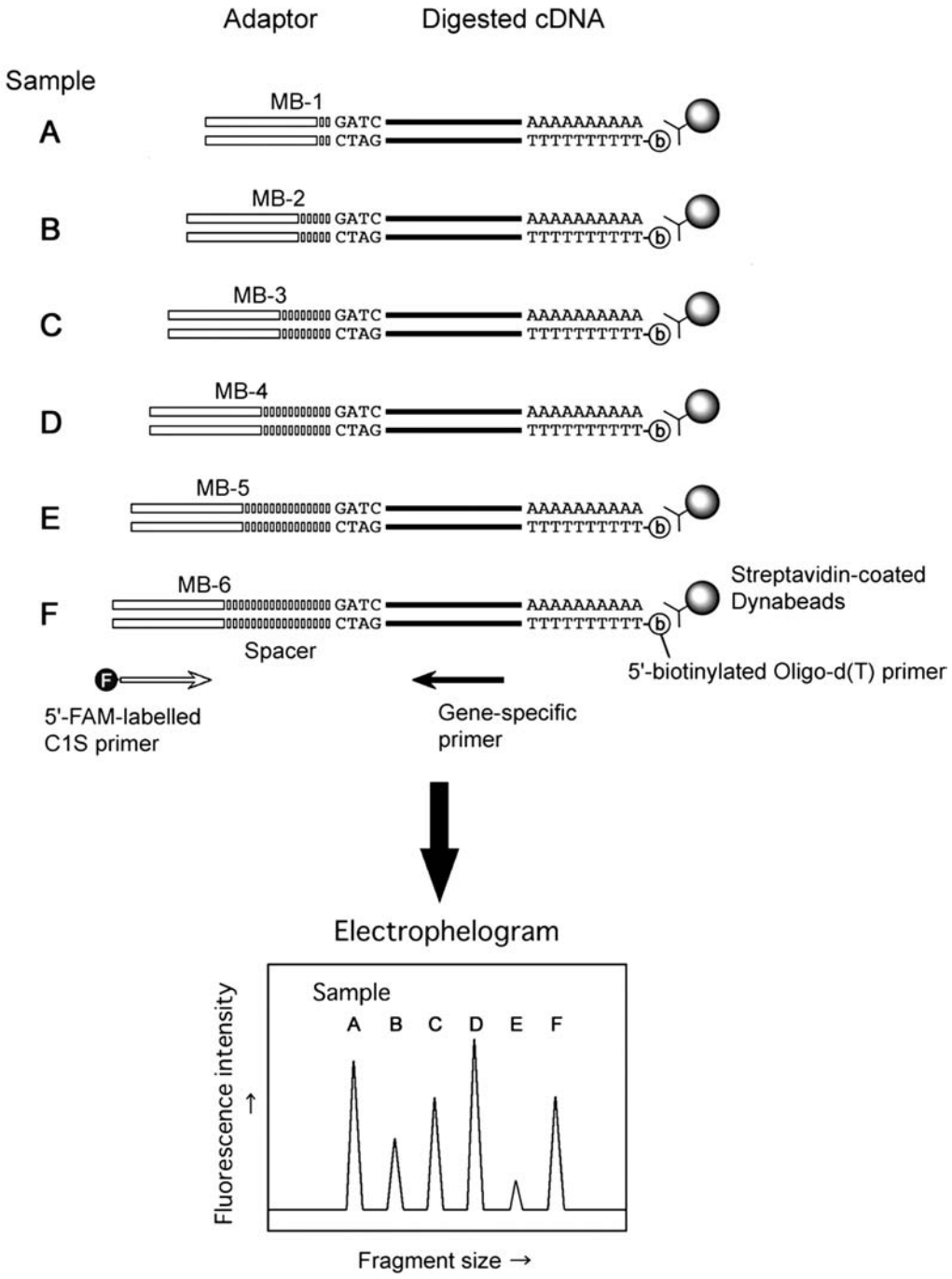

Fig. 1. The principle of ATAC-PCR. This schematic diagram depicts ATAC-PCR used on *Mbo*I-digested cDNA samples.

in Fig. 1. Each cDNA is prepared from total RNA, digested with a restriction enzyme, and then ligated to an adapter using the cohesive end created enzymatically. Multiple adapters sharing a common sequence but featuring spacer regions of different sizes are normally used (Fig. 2). After mixing the adapter-ligated samples in a single tube, PCR amplification is performed; a fluorescently labeled adapter primer corresponding to the common region is used in conjunction with a gene-specific primer. PCR products are separated by polyacrylamide gel electrophoresis and those from different samples can be distinguished by spacer region size. The yield from each product reflects the amount of original template cDNA, and relative sample expression levels can be calculated from their signal intensities.

Six different adapters are typically attached to cDNA samples in a single experiment. Fig. 3 shows an example of ATAC-PCR using six adapter primer/cDNA combinations. Three adapters were used to label control cDNA samples for calibration purposes. A control

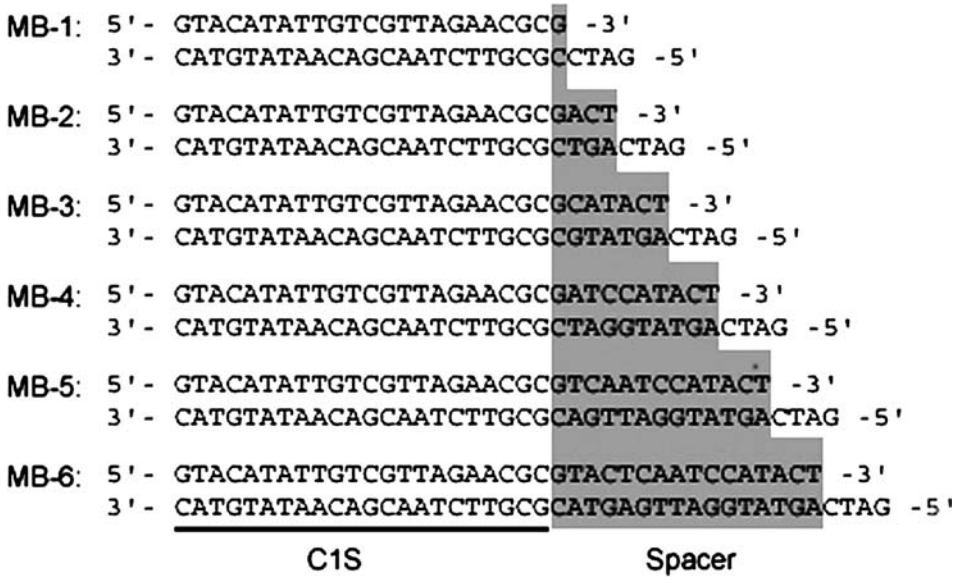

Fig. 2. The sequences and unique sizes of the six adapters.

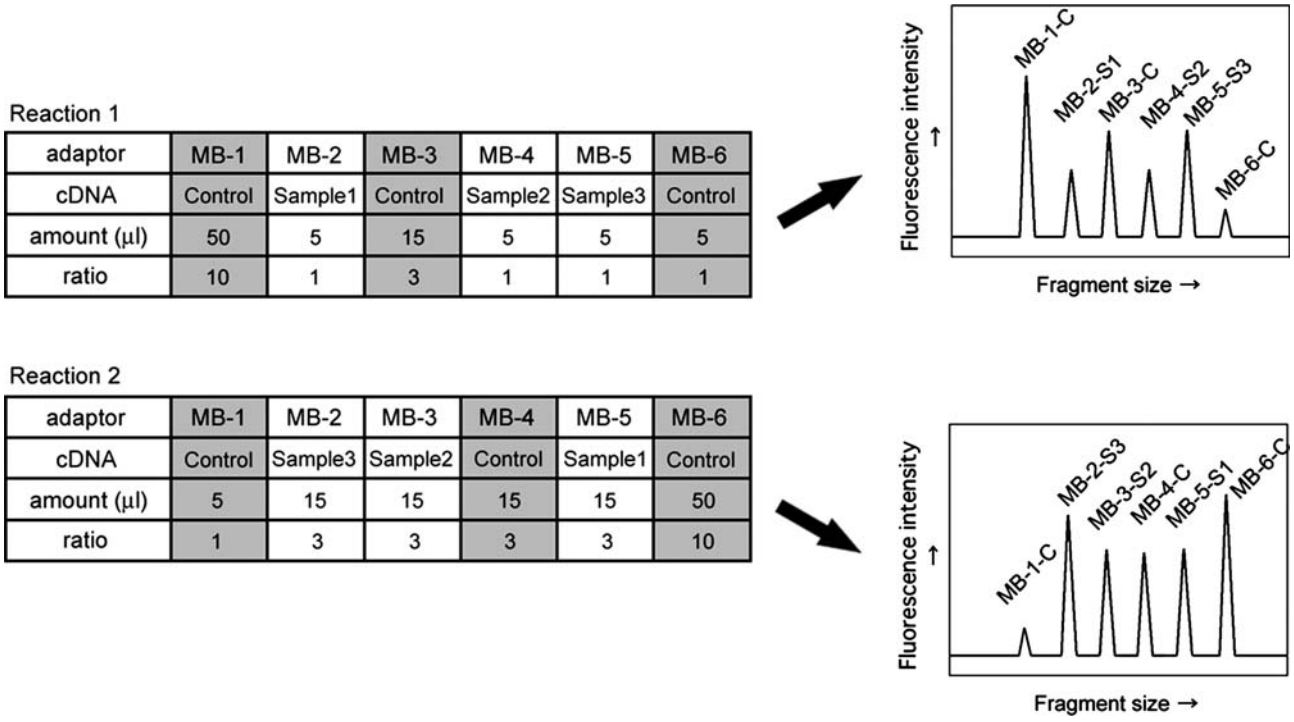

Fig. 3. An example in which two combinations of the six adapters and cDNAs are used for ATAC-PCR.

cDNA prepared from total RNA was ligated to three adapters of different lengths and these were mixed at a ratio of 1:3:10. At the same time, three cDNAs from sample tissues (sample cDNAs) were ligated to another set of adapters of different lengths and mixed in equal proportions. The final mixture of all six cDNAs was subjected to PCR amplification in a single reaction tube.

To ensure that data points for sample RNAs were covered within the range of calibration, two different assays with distinct calibrations were performed (Fig. 3). One calibration used the control samples mixed at a ratio of 1:3:10 tagged with three different adapters (MB-1, MB-3, and MB-6) together with test samples tagged with three other adapters (MB-2, MB-4, and MB-5) in onefold amounts each. The other calibration used control samples mixed at a ratio of 10:3:1 tagged with a different set of adapters (MB-1, MB-4, and MB-6) together with test samples tagged with the remaining three

adapters (MB-2, MB-3, and MB-5) in threefold amounts each. These two calibration curves usually permit quantification within a reasonable range.

3. Materials and methods

We describe here the use of ATAC-PCR with the restriction enzyme *Mbo*I. The ATAC-PCR protocol is essentially the same as described previously [6,12].

3.1. Primer design

For each gene, 20–25 bp gene-specific primers were designed to amplify 70–150 bp-long fragments by ATAC-PCR. As ATAC-PCR uses the 3′-end sequences of cDNA, databases of 3′-directed cDNA sequences such as BodyMap (http://bodymap.ims.u-tokyo.ac.jp) and our own ([7], http://www.love2.aist-nara.ac.jp) are especially useful.

3.2. Preparation of MboI-digested cDNA

Three micrograms of total RNA was converted to cDNA in a 20 μl reaction mixture with 15 pmol of both 5′-biotinylated oligo$(dT)_{18}$ primer and Superscript II reverse transcriptase (Invitrogen). cDNA, which had been synthesized as described previously [12], was then digested with 40 U *Mbo*I (TaKaRa) at 37 °C for 1 h, in a 1× K buffer (20 mM Tris–HCl, pH 8.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 mM KCl) and in a reaction volume of 400 μl. Digested cDNA was purified with phenol–chloroform, precipitated in 70% ethanol, and resuspended in 1.2 ml of 0.1× TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA · 2Na, pH 8.0).

3.3. Adapter ligation and PCR amplification

Twenty microliters of each *Mbo*I-digested cDNA sample was mixed with 30 pmol of one of the six adapters, 3 μl of 10× T4 ligation buffer (300 mM Tris–HCl, pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, and 10 mM ATP) and 3 U T4 DNA ligase (Promega) in a final volume of 30 μl. After incubation at 16 °C overnight, the six different ligation mixtures were combined and the 3′-end cDNA fragments were recovered with streptavidin-coated beads (3.0 mg/ml Dynabeads M-280 Streptavidin (Dynal) in 1.0 M NaCl). The cDNA fragment mixture was then washed with distilled water and suspended in 300 μl of the same for use as a PCR template.

3.4. ATAC-PCR

The 5 μl ATAC-PCR mixture contains 3′-end cDNA fragments (obtained from 2 ng of each total RNA) as well as 0.5 μl of 10× PCR buffer (40 mM Tris–HCl, pH 8.9, 3 mM $MgCl_2$, 50 mM KCl, and 200 μg/ml gelatin), 0.5 nmol of each dNTP (dATP, dCTP, dGTP, and dTTP), 0.25 U AmpliTaq Gold DNA polymerase (PE Applied Biosystems), and 0.5 pmol of both a gene-specific primer and a carboxyfluorescein (FAM)-labeled C1S primer (5′-GTACATATTGTCGTTAGAACGC-3′). The reaction mixture was preincubated for 10 min at 95 °C to activate the enzyme and then subjected to 40 rounds of a PCR cycle (20 s at 94 °C, 40 s at 50 °C, and 40 s at 72 °C), followed by incubation at 72 °C for 20 min. ATAC-PCR were typically performed in 96- or 384-well microtiter plates.

3.5. Electrophoresis and data analysis

The fluorescence intensity of each PCR product was measured with a capillary type sequencer, such as the ABI3100 genetic analyzer or the ABI3700 DNA analyzer (Applied Biosystems). Each 5 μl of reaction mixture was mixed with 0.2 U of T4 DNA polymerase (TaKaRa) and incubated at 37 °C for 2 h to blunt the PCR products. Approximately 1.0–2.5% of the PCR products was admixed with a GeneScan-500 Rox size standard (Applied Biosystems) and applied to the capillary sequencer. Fragment size and fluorescence intensity were analyzed using GeneScan analysis software (Applied Biosystems). One can also substitute ordinary polyacrylamide gel electrophoresis together with a densitometer for this equipment.

4. ATAC-PCR assay using LCM samples

In the developing mouse cerebellum, distinct stages in granule neuron development are defined by changing patterns of gene expression [13]. The mRNAs of several genes differentially localize to the proliferating granule cells in the superficial layer of the external granule layer (EGL), the postmitotic and migrating granule cells at the internal EGL surface, and the mature granule cells at the internal granule layer (IGL). Using a PixCell LCM microscope (Arcturus Engineering), the superficial layer of the EGL, the internal surface of the EGL, and the IGL were dissected separately from stained 12-μm frozen tissue sections of postnatal day 12 mouse cerebellum (Fig. 4). The protocols for microdissection and RNA preparation can be found on the NIH LCM web page (http://dir.nichd.nih.gov/lcm/lcm.htm). Approximately 100 ng of total RNA could be obtained from 1000 transfer shots of the EGL and the IGL. Five hundred nanogram aliquots of total RNA purified from LCM samples were converted to cDNA and 3′-end cDNA fragments (originating from 2 ng of each total RNA) were used for ATAC-PCR amplification.

Fig. 5 shows the results of quantifying $GABA_A$ receptor α6 subunit mRNA from LCM samples by ATAC-

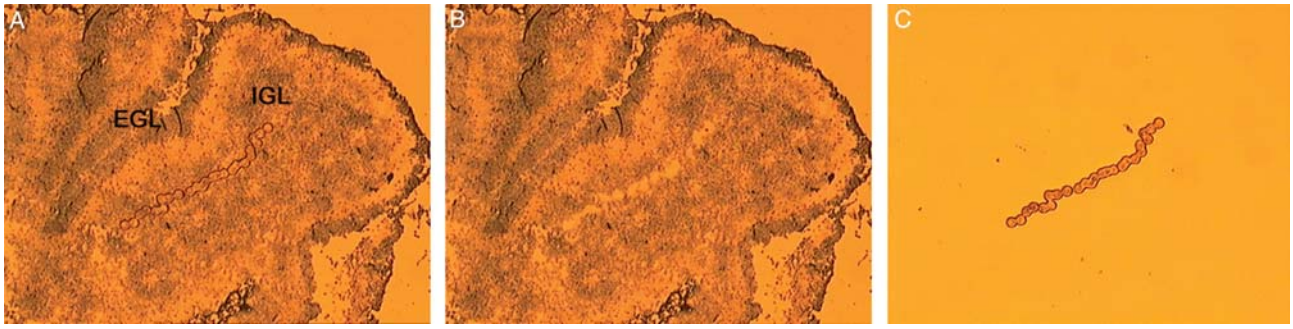

Fig. 4. LCM capture of the granule layer from sections of postnatal day 12 mouse cerebellum. The tissue was fixed in ethanol and stained by hematoxylin and eosin (HE). (A) The IGL is targeted with individual laser shots. Each 35-μm-diameter shot is seen as a circle. EGL, external granule layer; IGL, internal granule layer. (B) The tissue section after LCM. The targeted tissue has been removed from the section. (C) Transfer film surface following LCM.

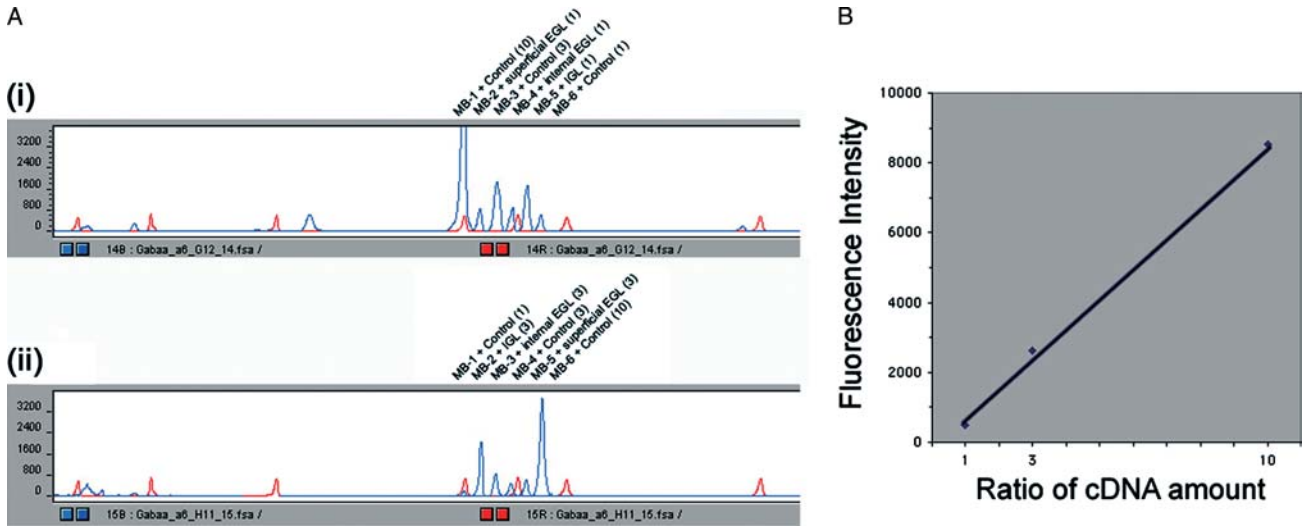

Fig. 5. Quantification by ATAC-PCR of the $GABA_A$ receptor α6 subunit gene transcript in the superficial layer of the EGL, the internal surface of the EGL, and the IGL. (A) The ATAC-PCR electropherogram. Total RNA from cerebrum at postnatal week 6, used as a control, was prepared using Trizol (Invitrogen). For quantification of relative expression levels, cDNA prepared from week 6 cerebrum was ligated to three adapters of different lengths and these were mixed at a ratio of 1:3:10. Three cDNAs from other samples were ligated to another set of adapters of different lengths mixed at a ratio of 1:1:1 relative to the standards, and combined with the three standard cDNAs. (i) For a differently calibrated assay, cDNA prepared from week 6 cerebrum was labeled with a different adapter set and mixed at a ratio of 10:3:1. Three sample cDNAs were ligated to the remaining three adapters of different lengths mixed at a ratio of 3:3:3 relative to the standards, and combined with the three standard cDNAs. (ii) The combined mixture of six cDNA samples was subjected to PCR in a reaction tube with a gene-specific primer (5′-AGTACACAAGGTTGAATCCT-3′) for $GABA_A$ receptor α6. The fluorescence intensity of the PCR product was measured with an ABI3100 genetic analyzer (Applied Biosystems). (B) The calibration curve constructed from the fluorescence intensities of the three standard samples is shown in A-(i). Relative expression levels of the $GABA_A$ receptor α6 gene in the EGL and IGL were estimated with this calibration curve.

PCR. Total RNA from cerebrum at postnatal week 6 was used as a standard. Based on the calibration curve shown in Fig. 5B, the expression levels of the $GABA_A$ receptor α6 subunit gene in the superficial layer of the EGL, the internal surface of the EGL, and the IGL were calculated as 1.2-fold, 1.5-fold, and 3.1-fold higher, respectively, than those found in the 6-week cerebrum.

5. Concluding remarks

ATAC-PCR is a variant of competitive quantitative PCR that is used to measure the gene expression ratio of multiple samples in a single PCR. ATAC-PCR is highly sensitive and can identify small differences in gene expression. Using multiple adapters for this assay, the expression levels of hundreds of genes can be analyzed simultaneously from only a few micrograms of total RNA. This technique provides valuable confirmation of microarray results and can facilitate large-scale gene expression analysis as well.

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