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A relative quantitative method to detect *OCT4A* gene expression by exon-junction primer and locked nucleic acid-modified probe*

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Abstract: Objective: OCT4A has been known to play a critical role in the maintenance of pluripotency of embryonic stem cells. Recent research has shown that OCT4A is also expressed in partial tumor cell lines and tissues. This study is aimed to develop a real-time reverse transcriptase polymerase chain reaction (RT-PCR) assay for relative quantitative detection of *OCT4A* mRNA and discrimination from *OCT4B*, pseudogene, and genomic contaminations. Methods: A locked nucleic acid (LNA)-modified probe was designed to discern the single base difference 352A/C to identify *OCT4A* mRNA. An exon-junction primer was designed to avoid false positive caused by genomic contaminations. In addition, a house keeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was measured in parallel to normalize the differences between samples and operations. Results: Experiments showed that the newly established RT-PCR assay amplified the *OCT4A* mRNA selectively; *OCT4A* analogues gave negative signals. Cell lines nTERA-2 and HepG2 showed positive results in *OCT4A* expression, while for HeLa and 293 cell lines, as well as primary peripheral blood mononuclear cells (PBMCs), *OCT4A* expression was negative. Additionally, the relative quantity of *OCT4A* mRNA was calculated by cycle threshold (C_t) method and house keeping gene normalization. Conclusions: This technique proved to be effective for relative quantitation of *OCT4A* mRNA with high specificity.

Key words: *OCT4A*, Locked nucleic acid (LNA), Real-time polymerase chain reaction (PCR)
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1 Introduction

Octamer-4 (Oct-4A) is a member of the POU family of transcription factors and is expressed in both embryonic stem (ES) cells and embryonal carcinoma (EC) cells. When cells are driven into embryonic and extraembryonic somatic differentiation, they lose Oct-4A expression (Takeda *et al*., 1992; Preston *et al*., 2003; Zangrossi *et al*., 2007). The human *Oct-4* gene, located on chromosome 6p21.31, is alternatively spliced, encoding *OCT4A* and *OCT4B*. OCT4A confers the stemness properties of ES cells and is critically involved in the self-renewal of undifferentiated ES cells, whereas the function of OCT4B is unknown (Scholer *et al*., 1989; Kerkis *et al*., 2006; Medvedev *et al*., 2008). The basic local alignment search tool (BLAST) search results showed that the coding region of *OCT4A* exon 1 had high homology with human *POU5F1B* mRNA. The sequence homologous rate of nucleotide was 96%. At genomic level it also had high homology with fragments of chromosomes 1 and 12, which seem as *OCT4A* pseudogene. The homologous rates were 95% and 97%, respectively. Recent reports have described OCT4A expression in several human tumor cell lines. Reactivation of OCT4A expression is postulated to occur in differentiated cells that have undergone carcinogenesis, or tumor formation

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(Looijenga *et al*., 2003; Suo *et al*., 2005; Cantz *et al*., 2008). To resolve the apparent discrepancy in OCT4A expression between earlier and recent studies concerning whether OCT4A is specifically expressed in ES cells and in tumor cells, but not in cells of differentiated tissues, we designed a method to differentiate between *Oct-4A* and *Oct-4B*, *OCT4A* pseudogene expression at mRNA level. In addition, the method can avoid false positive caused by contaminated genomic DNA.

2 Materials and methods

2.1 Principle of quantitative measurement of *OCT4A* expression

OCT4A has 5 exons and 4 introns, and only exon 1 is special. Its exons 2, 3, 4, and 5 are exactly the same as *OCT4B* (Fig. 1). Sequence analysis has shown that the coding region of *OCT4A* exon 1 contains some special sites, namely 234C and 352A, which are different from *POU5F1B* mRNA and other *OCT4A* pseudogene sequences (Fig. 2). This difference was exploited to create assays selective for *OCT4A* expression. Our strategy is shown in Fig. 3. Firstly, a pair of primers was designed. Forward primer P1 was complementary to *OCT4A* exon 1. Reverse primer was design as exon-bridge primer P2, which is composed of two segments (segment A near 3′ end is complementary to *OCT4A* exon 1 end sequence, segment B near 5′ end is complementary to *OCT4A* exon 2 beginning sequence). Thus, in reverse transcriptase polymerase chain reaction (RT-PCR) reaction system (Nolan *et al*., 2006), exon-bridge primer only pairs with transcript mRNA, and the genomic sequence cannot be amplified. In addition, a fluorogenic locked nucleic acid (LNA)-modified probe P3 (FAM-caggggTGacggtg-BHQ1) was designed to identify base divergence 352A/C site, which can differentiate *OCT4A* mRNA from *OCT4B* and *OCT4A* pseudogene expressions. *Taq* polymerase 5′-nuclease activity enables the system to amplify fluorescence with the accumulation of specific PCR product. Real-time quantitative PCR eliminates the variability associated with conventional PCR, thus allowing routine and reliable quantitation of nucleotide templates. In order to test and verify this novel strategy, we also designed another two pairs of primers, which were used in conventional PCR and RT-PCR as comparison. One primer set was composed of exon-bridge primer P2 and forward primer P1, another primer set was composed of P4 and P5, each of which was complementary to *OCT4A* exon 1 region. These primer sites and sequences are listed in Table 1.

Table 1 Primer and probe sequences

Primer and probe names	Sequence
P1	gcttggagacctctcagcct
P2	ttgatgtcctgggactcctc
P3	FAM-caggggT$^{\#}$G$^{\#}$acggtg-BHQ1
P4	TCGGACCTGGCTAAGCTTCC
P5	TGCTCCAGCTTCTCCTTCTCC
H-*GAPDH*-1	TGAGCACCAGGTGGTCTCC
H-*GAPDH*-2	TAGCCAAATTCGTTGTCATACCAG
H-*GAPDH*-3	FAM-ACAGCGACACCCACTCCTCCACC-BHQ1

$^{\#}$ Locked nucleic acid-modified site

2.2 Construct simulation models of *OCT4A*, *OCT4B*, and two *OCT4A* pseudogenes

Clones of *OCT4A*, *OCT4B*, and two *OCT4A* pseudogenes were constructed by a two-step PCR-based gene synthesis method. Based on sequence in GenBank data, we synthesized overlapping oligonucleotides which, when assembled, formed the template for the gene of interest (Fig. 4).

2.2.1 Gene assembly and amplification

We mixed the equal volumes of oligonucleotide solutions, which were then diluted with water to get a final concentration of ~1 ng/μl for each. Diluting the oligonucleotide mixture with PCR solution for five-fold. The final concentrations of components were 0.2 ng/μl for each oligonucleotide, 20 mmol/L for Tris-HCl (pH 8.8), 10 mmol/L for KCl, 10 mmol/L for $(NH_4)_2SO_4$, 6 mmol/L for $MgSO_4$, 0.1% (v/v) for Triton X-100, 0.1 mg/ml for bovine serum albumin, 0.2 mmol/L for each dNTP, and 2.5 U for Pfu polymerase. In the PCR protocol, we added the polymerase to avoid any possible mispriming, which meant hot start PCR in gene assembly, which began with one 5-min denaturation step of 95 °C, then 25 cycles of denaturation temperature 95 °C for 30 s, variable annealing temperature for 30 s, and extension temperature of 72 °C for 1.5 min. In the

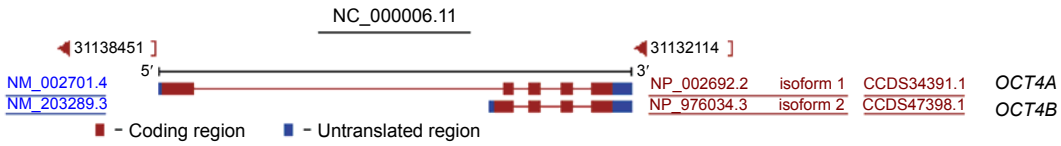

Fig. 1 Gene structures of *OCT4A* and *OCT4B*

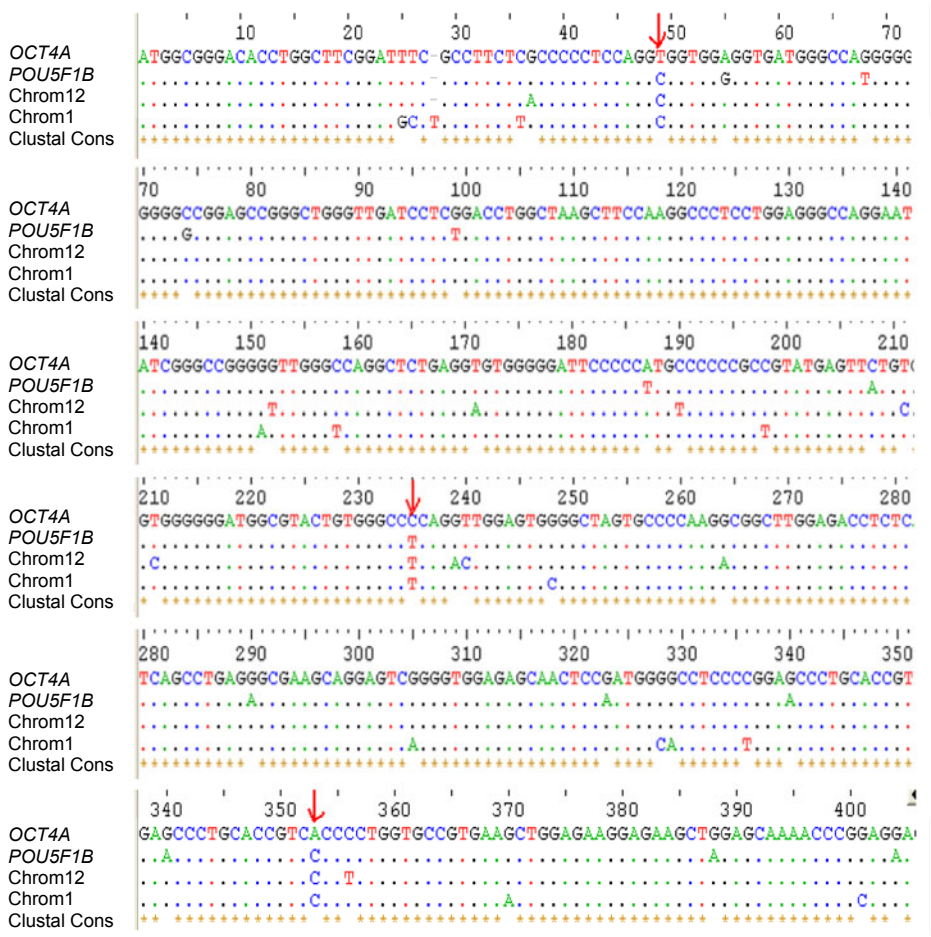

Fig. 2 Sequence alignment of the coding region of human *OCT4A* exon 1 with its highly homologous sequence

The coding region of *OCT4A* exon 1 had high homology with human *POU5F1B* mRNA and fragments of chromosomes 1 and 12. Red arrow shows the key different sites

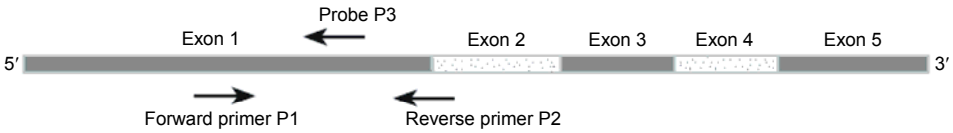

Fig. 3 Positions of PCR primers and probes used for human *OTC4A* detection

Exons 1, 2, 3, 4, 5 are indicated. The forward primer is on exon 1, which is between CDS 266 and 285; the reverse primer is spanning the junction between exons 1 and 2, and has six base pairs on exon 1 at the 3′ end, which is between CDS 419 and 400; the probe is on exon 1, which has a special 352A site different from *POU5F1B* and other similar genes, which are all 352C

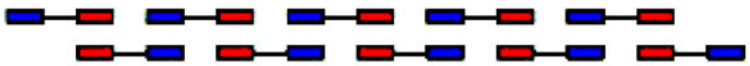

Fig. 4 Gene assembly principle

Synthesized overlapping oligonucleotides were assembled by complementary base pairing. The blue and red boxes represent complementary nucleotide bases, respectively

PCR protocol, the last step was an incubation cycle at 72 °C for 10 min. The template will be 1 μl of the mixture resulting from the gene assembly reaction, and the primer will be the outermost oligonucleotides for the gene amplification. In the PCR protocol, the gene amplification is the same as gene assembly in essence, and only the annealing temperature is different (raised to 62 °C).

2.2.2 Cloning and sequencing

We integrated the synthetic gene fragments which were purified by gel extraction into the vector using the TA cloning system. The ligation products were transformed into DH5α *Escherichia coli* cells and selected on Luria-Bertani (LB) medium plates with 50 μg/ml ampicillin. The plasmids isolated were screened by PCR using primers complementary to vector sequences flanking the synthetic gene, and plasmids containing the gene of interest were sequenced in both the forward and reverse directions.

2.2.3 In vitro transcription

After clones of interest were validated, they were linearized by restriction enzyme digestion, followed by double extraction with phenol: chloroform (1:1, v/v) and single extraction with chloroform. Sodium acetate (0.3 mol/L) was added and nucleic acid was precipitated with 0.75 ml of isopropanol. The precipitate was dissolved in 100 μl TE solution (10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L ethylenediaminetetraacetic acid (EDTA)). T7 transcription kit was used to amplify the target gene RNA. The procedures were largely based on the vendor's protocol.

2.3 Sample preparation

Cell lines nTERA-2, HepG2, HeLa, and 293 were obtained from the ATCC. Cells were thawed and plated into six-well culture dishes (Corning, Shanghai, China) without a feeder layer. The growth medium used was Dulbecco's modified Eagle medium (DMEM). Cells were cultured at 37 °C and 5% CO_2, and the medium was replaced every 24 h. Cultured cells (5×10^5–1×10^6 cells) were collected by trypsinization and washed with phosphate-buffered saline (PBS). Peripheral blood mononuclear cells (PBMCs) were isolated by FICOLL (Sigma, Shanghai, China).

2.3.1 RNA extraction

A total of 1 ml of TRIzol was added to a cell pellet. After resuspending the cells, the tubes were vigorously shaken at room temperature for 1 min. Immediately after, the samples were added to 200 μl chloroform, shaken, and stored at room temperature for 5 min; the extraction mix was centrifuged for 15 min at 12000×*g* in 4 °C to promote phase separation. The aqueous phase was then retrieved and mixed with an equal volume of isopropanol, incubated at room temperature, and centrifuged to concentrate the precipitated RNA. The RNA pellet was washed using 75% ethanol, then air-dried, and finally dissolved in diethylpyrocarbonate (DEPC) water.

2.3.2 Genome DNA extraction

Purification of cell genome DNA was carried out using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). Especially, RNase A should be added to the sample before addition of buffer AL (Qiagen). Other procedures were largely followed the vendor's protocol.

2.4 Relative quantification of *OCT4A* gene expression

2.4.1 Conventional PCR

Conventional PCR system was 50 μl volume reaction containing 5 μl 10× PCR reaction buffer, 0.5 μmol/L each forward and reverse primer, 200 nmol/L each of dATP, dTTP, dGTP, and dCTP, 50 nmol/L dUTP, 1.5 U HotStarTaq DNA polymerase (Qiagen), 0.5 U of uracil *N*-glycosylase (UNG), and 5 μl template. Cycle parameters were 37 °C for 2 min, 95 °C for 15 min, and 35 cycles of 94 °C for 15 s, 58 °C for 20 s, and 72 °C for 30 s. Reaction was carried out in a 9600 PerkinElmer apparatus (PerkinElmer, USA).

2.4.2 Conventional RT-PCR

Conventional RT-PCR system was 25 μl volume reaction containing 12.5 μl 2× RT-PCR reaction buffer, 0.5 μmol/L each forward primer and reverse primer, 200 nmol/L each dATP, dTTP, dGTP, and dCTP, 1.5 U HotStarTaq DNA polymerase, 200 U Super Script III reverse transcriptase, and 5 μl template. Cycle parameters were 45 °C for 15 min, 95 °C

for 15 min, and 35 cycles at of 94 °C for 15 s, 58 °C for 20 s, and 72 °C for 30 s.

2.4.3 Real-time fluorescence RT-PCR

Real-time fluorescence RT-PCR system was 20 μl volume reaction containing 10 μl 2× real-time RT-PCR reaction buffer, 0.5 μmol/L primer P1 and P2, 0.25 μmol/L of TaqMan LNA probe, 200 nmol/L each dATP, dTTP, dGTP, and dCTP, 1.5 U Hot-StarTaq DNA polymerase, 200 U Super Script III reverse transcriptase, and 2 μl template. Cycle parameters were 45 °C for 15 min, 95 °C for 15 min, and 40 cycles at 94 °C for 5 s and 60 °C for 25 s. Fluorescence value of each tube was measured at the end of 60 °C step. House keeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was measured in parallel, which was used as the internal control to normalize the difference (Kheirelseid *et al*., 2010).

3 Results

3.1 Construct simulation models of *OCT4A*, *OCT4B*, and two *OCT4A* pseudogenes

Sequencing data (in both the forward and reverse directions) validated that four gene fragments had been cloned into the PUCm18 T vectors separately. These clones were named as PUCm18-*OCT4A*, PUCm18-*OCT4B*, PUCm18-*OCT4a1*, and PUCm18-*OCT4a2*. These four inserted sequences were compared by software ClustalX 1.81 and BioEdit 7.09. *OCT4A* exon 1 had three special sites 48T, 234C, and 352A, different from other three sequences. These plasmids were diluted in 1:100000 with TE solution, and used as templates in the following PCR reaction. After in vitro transcription, the products for each plasmid were diluted in 1:100000 by DEPC pure water, and then were used as templates in the next RT-PCR reaction.

3.2 Specificity of the method for *OCT4A* expression

To evaluate whether the novel strategy can detect selectively *OCT4A* expression from other similar forms, namely *OCT4B* and *OCT4A* pseudogenes, conventional PCR and RT-PCR were used for comparison. Conventional PCR (P1, P2 as primer sets) showed that genomic DNA as template gave a negative result (Fig. 5b), meanwhile they were positive (Fig. 5a) in another conventional PCR (P5, P6 as primer sets). It means that the junction primer P2 contributed to avoiding the false positive caused by genomic DNA. This hypothesis went a step further. In conventional RT-PCR (P1, P2 as primer sets), nTERA-2, HepG2, HeLa cell RNA extractions gave a positive result, PBMC and 293 cells were negative (Fig. 5d). However, in another RT-PCR (P5, P6 as primer sets), these cell RNA showed weak positive (Fig. 5c). It means that the amplification products of HepG2 and HeLa cells in conventional RT-PCR (P1, P2 as primer sets) were really from transcription RNA, not contaminated genomic DNA. Furthermore, among four simulation transcription models, only *OCT4A* model gave a positive signal (Fig. 6a) in real-time RT-PCR (P1, P2 as primers; P3 as probe). Considering other experiment (Fig. 5d), it meant that LNA-modified probe discriminate *OCT4A* mRNA from other similar transcription forms. In addition, we found that HeLa cell RNA gave a negative result in real-time RT-PCR, while nTERA-2 and HepG2 were positive (Fig. 6b).

3.3 Relative quantification of *OCT4A* gene expression

Relative quantification is the most widely adopted approach. Quantification of gene expression is based on the analysis of a target gene whose expression is normalized relative to the expression of a control gene. In our study, *GAPDH* gene was used as house-keeping gene. *OCT4* gene relative quantization data are shown in Table 2.

Table 2 *OCT4A* mRNA relative quantification with comparative cycle threshold (C_t) method

Cells	Average *OCT4A* C_t	Average *GAPDH* C_t	ΔC_t[a]	$\Delta\Delta C_t$[b]	*OCT4A*/nTERA-2[c]
nTERA-2	26.85±0.15	19.76±0.07	7.09±0.18	0.00±0.18	1
HepG2	29.71±0.17	20.32±0.10	9.39±0.21	2.30±0.21	0.176–0.235
293	ND	21.67±0.09	−	−	−
HeLa	ND	19.35±0.09	−	−	−

Data are expressed as mean±standard deviation (SD). [a] The ΔC_t value was determined by subtracting the average *GAPDH* C_t value from the average *OCT4A* C_t value (C_t (*OCT4A*)− C_t (*GAPDH*)); [b] The calculation of $\Delta\Delta C_t$ was arbitrary constant, so the standard deviation of $\Delta\Delta C_t$ was same as the standard deviation of ΔC_t (ΔC_t (*OCT4A*)−ΔC_t (nTERA-2)); [c] The range given for *OCT4A* mRNA relative to nTERA-2 cell line was determined by evaluating the expression: $2^{-\Delta\Delta C_t}$ with $\Delta\Delta C_t$±SD. ND: undetectable; −: negtive

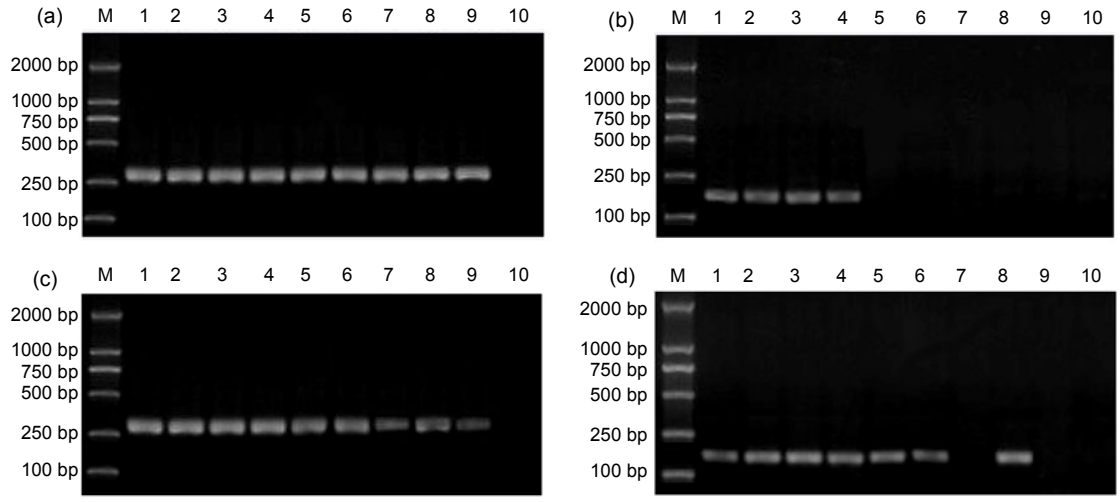

Fig. 5 Conventional PCR and RT-PCR for *OCT4A* expression

(a) Conventional PCR (P5, P6 as primer sets); (b) Conventional PCR (P1, P2 as primer sets). Lanes 1–4: plasmid dilution as PCR amplification templates. They were PUCm18-*OCT4A*, PUCm18-*OCT4B*, PUCm18-*OCT4a1*, and PUCm18-*OCT4a2*, respectively; Lanes 5–9: genomic DNA extraction as PCR templates. They were nTERA-2, HepG2, 293, HeLa, and PBMC, respectively; (c) Conventional RT-PCR (P5, P6 as primer sets); (d) Conventional RT-PCR (P1, P2 as primer sets). Lanes 1–4: in vitro transcription RNA as RT-PCR amplification templates. They were from plasmid PUCm18-*OCT4A*, PUCm18-*OCT4B*, PUCm18-*OCT4a1*, and PUCm18-*OCT4a2*, respectively. Lanes 5–9: RNA extraction as RT-PCR templates. They were nTERA-2, HepG2, 293, HeLa, and PBMC, respectively

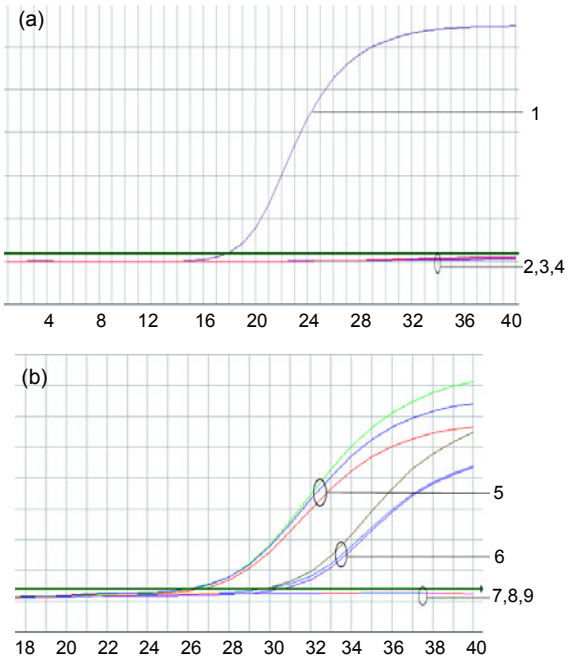

Fig. 6 Real-time RT-PCR for *OCT4A* and *OCT4B* expressions in different samples (P1, P2 as primers; P3 as probe)

1: PUCm18-*OCT4A* in vitro transcription RNA as real-time RT-PCR amplification template; 2–4: in vitro transcription RNA as real-time RT-PCR amplification templates. They were from plasmid PUCm18-*OCT4B*, PUCm18-*OCT4a1*, and PUCm18-*OCT4a2*, respectively; 5–9: RNA extraction as RT-PCR templates. They were nTERA-2, HepG2, HeLa, 293, and PBMC, respectively

4 Discussion

The ability to relatively quantify mRNA represents a major step forward in tissue transcript analysis, but for *OCT4A* gene, the key and the greatest difficulty is to distinguish the false appearances from pseudogenes, other transcripts, and genomic contaminations. It is really important to detect the *OCT4A* mRNA accurately for learning the expression state for OTC4A in tissues, cell lines, and many kinds of tumor and series of cell differentiation stage. It is an important method of defining the function of this gene. Detection of single base substitution using a high affinity DNA analogue known as LNA for allelic discrimination assays has been achieved by various methods (Braasch and Corey, 2001; Petersen *et al*., 2003). Real-time PCR has been investigated as a platform to perform SNP genotyping with the utilization of LNA/DNA duplexes (Johnson *et al*., 2004).

Based on the results, we found that using exon-junction primer can avoid the false positive result caused by the genomic contaminations in the process of RNA sample extraction. LNA probe can discriminate the *OCT4A* mRNA and *OCT4B* mRNA and pseudogenes mRNA at a transcript level.

Moreover, adopting a house keeping gene as

internal control, it can revise the cell amount difference among the samples and the nucleic acid extraction yield difference. We can then study the *OCT4A* mRNA relative expression amount among different tissues and cell samples. In summary, our method is capable of detecting the *OCT4A* expression at a quantitative level, thus facilitating further study of OCT4A function.

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