USM J/P-06

BAHAGIAN PENYELIDIKAN & PEMBANGUNAN

CANSELORI

UNIVERSITI SAINS MALAYSIA

Laporan Akhir Projek Penyelidikan Jangka Pendek

1) Nama Penyelidik: .....Dr. Nik Soriani Yaacob .....

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Nama Penyelidik-penyelidik
Lain *(Jika berkaitan)* : .....Prof. Madya Dr. Norazmi Mohd. Nor.....

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2) Pusat Pengajian/Pusat/Unit: .....PP Sains Perubatan.....

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3) Tajuk Projek: .....Gene quantitation by a competitive quantitative PCR technique

.....using a homologous standard.....

4) (a) Penemuan Projek/Abstrak

(Perlu disediakan makluman di antara 100 – 200 perkataan di dalam Bahasa Malaysia dan Bahasa Inggeris. Ini kemudiannya akan dimuatkan ke dalam Laporan Tahunan Bahagian Penyelidikan & Pembangunan sebagai satu cara untuk menyampaikan dpatan rojek tuan/puan kepada pihak Univesiti).

The project aimed to develop a quantitative PCR technique to accurately quantify gene expression. We tested the accuracy of the method by determining the expression levels of the transcription factor, PPARγ. This was done firstly in a mock system (utilizing cloned PPARγ in a plasmid with known concentrations) and later on activated monocytes. The method utilized a homologous internal standard in a competitive PCR. This method has the advantage over using housekeeping genes as control since the latter requires different primers and amplification conditions. The current method also has advantage over using PCR MIMIC which may have different amplification efficiency due to the different gene fragment used. The homologous standard was constructed from the same target gene with a slightly shorter length to allow for visualization and analysis following competition with the target gene. The mock experiment showed that the method worked successfully. Using cDNA prepared from activated monocytes we were also able to show that it is useful for determining changes in the levels of PPARγ expression in cells expressing the transcription factor.

Projek ini bertujuan membangunkan kaedah yang tepat bagi kuantifikasi ekspresi gen tertentu. Ketepatan kaedah ini diuji melalui pengukuran tahap ekspresi faktor transkripsi, PPARγ. Ini dilakukan pada mulanya melalui suatu ujian cubaan (dengan menggunakan plasmid yang mempunyai gen tersebut dalam kepekatan yang diketahui) dan kemudiannya ke atas monosit teraktif. Kaedah ini menggunakan standard internal yang homologus. Kaedah ini mempunyai kelebihan berbanding penggunaan gen "housekeeping" , yang menggunakan primer dan parameter PCR yang berlainan. Ia juga mempunyai kelebihan berbanding PCR MIMIC yang mungkin mempunyai efisiensi PCR yang berlainan kerana penggunaan gen yang berlainan. Kaedah yang digunakan dalam projek ini memastikan parameter amplifikasi yang hampir identikal dengan gen sasaran. Standard homologus yang dimaksudkan dibangunkan daripada gen sasaran tetapi pendek sedikit untuk membolehkannya dianalisa selepas proses PCR kompetitif. Ujian awal menggunakan ujian cubaan menunjukkan bahawa kaedah ini dapat dijalankan dengan jayanya. Kami juga mampu menunjukkan bahawa pebezaan tahap ekspresi PPARγ dapat dikesan dalam monosit teraktif.

(b) Senaraikan Kata Kunci yang digunakan di dalam abstrak:

Bahasa Malaysia	Bahasa Inggeris
PCR kuantitatif kompetitif	Quantitative competitive PCR
Standard internal homologus	Homologous Internal standard
PPARγ.....	PPARγ.....
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.....	.....

5) Output Dan Faedah Projek

(a) Penerbitan (termasuk laporan/kertas seminar)
(Sila nyatakan jenis, tajuk, pengarang, tahun terbitan dan di mana telah diterbit/dibentangkan).

Ruzilawati A.B., Mohd. Ariffin K., Norazmi M.N. and Yaacob N.S.: Development of homologous internal standards for the quantitation of hPPARγ and mPPARγ using competitive PCR. . First ASEAN Conference on Medical Sciences, Renaissance Hotel Kota Bharu, 18 - 21 May, 2001.....
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(b) Faedah-faedah Lain Seperti Perkembangan Produk, Prospek Komersial Dan Pendaftaran Paten.
(Jika ada dan jika perlu, sila gunakan kertas berasingan)

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.....Tiada.....
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(c) Latihan Gunatenaga Manusia

i) *Pelajar Siswazah*:

.....Pn. Ruzilawati Abu Bakar *(Kursus Sarjana)*.....
.....(PP Sains Perubatan) .....
.....
.....

ii) *Pelajar Siswazah:*

.....Zaihasra bt. Ismail.....
.....(PP Sains Kimia) .....
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.....

iii) *Lain-lain:*

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.....Tiada.....
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6. Peralatan Yang Telah Dibeli:

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UNTUK KEGUNAAN JAWATANKUASA PENYELIDIKAN UNIVERSITI

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Universiti Sains Malaysia
16150 Kubang Kerian
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TANDATANGAN PENGERUSI
JAWATANKUASA PENYELIDIKAN
PUSAT PENYELIDIKAN

LAPORAN AKHIR PROJEK USM JANGKA PENDEK

Gene quantitation by a competitive quantitative PCR technique using a homologous standard

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Schools of [1]Medical and [2]Health Sciences, USM Health Campus

USM Short Term Grant (304/PPSP/6131140)

Introduction

Gene quantitation is important for the detailed understanding of transcriptional activities of genes that are believed to play important roles in disease processes. Previous methods of gene quantitation are either semi-quantitative or require a lot of starting material for analysis (1-4). Since the advent of polymerase chain reaction (PCR), many researchers have attempted to utilize the power of PCR for gene quantitation. However, many problems have arisen from these early attempts due to the exponential nature of the amplification dynamics (5,6). Attempts were then made to include a standard which can be used to compare the amplification of the target with a known concentration of the standard. This included the use of housekeeping genes to provide relative quantitation with the assumption that such housekeeping genes do not change in the experimental condition (7). This however was not always the case. To overcome this problem, we and several workers have used standards such as the PCR MIMIC – a standard that has a homologous region whereby the same primers would be used to amplify the target and the standard (8). Thus begun the advent of competitive PCR for gene quantitation. However, the problem of using MIMIC is that the gene used is not homologous to the target gene hence raising the possibility of different amplification efficiencies between the target and standard. Hence a more reliable standard need to be developed to increase the reproducibility and accuracy of gene quantitation.

Objective

The main objective of this project was to develop a quantitative PCR technique to accurately quantify gene expression. To achieve the accuracy and consistency of the assay, we used an internal standard that is 100% homologous to, but shorter than the target gene. We tested the accuracy of the method by determining the expression levels of the transcription factor, peroxisome proliferator-activated gamma (PPARγ) in a mock system as well as in monocytes, a cell that is known to differentially express PPARγ during cellular activation.

Methodology

The target genes, hPPARα and PPARγ were previously amplified from the cDNA of human adipose tissue , a source with high expression of the PPARs. The amplified products of 660 bp for PPARα and 580bp for PPARγ were then cloned into the plasmid vector, pCR.TOPO2.1 to produce the recombinant plasmids pNSY 001 and pNSY 004, respectively. For the current project, the hPPARα and hPPARγ inserts were excised from the recombinant plasmids above and recloned intothe plasmid vectors, pBlueScript II KS+ and pSG5, respectively (Figures 1 and 2). A small portion (about 70bp) was then removed from the middle part of each cloned insert by restriction digestion and the plasmid ligated back to produce pNSY 003 and pNSY 005 (Figure 3). These form the homologous standards to be used in subsequent experiments.

Serial 10-fold and 2-fold dilutions of the standards with known concentrations (determined spectrometrically) were used in a competitive PCR. Mock experiments were carried out using the original clones containing the PPARα or PPARγ gene as the target. A known concentration of the target was then run competitively with the serial dilutions of the homologous internal standard (2,9). To determine whether the technique can be used in a biological system, a similar experiment was then carried out on activated monocytes.

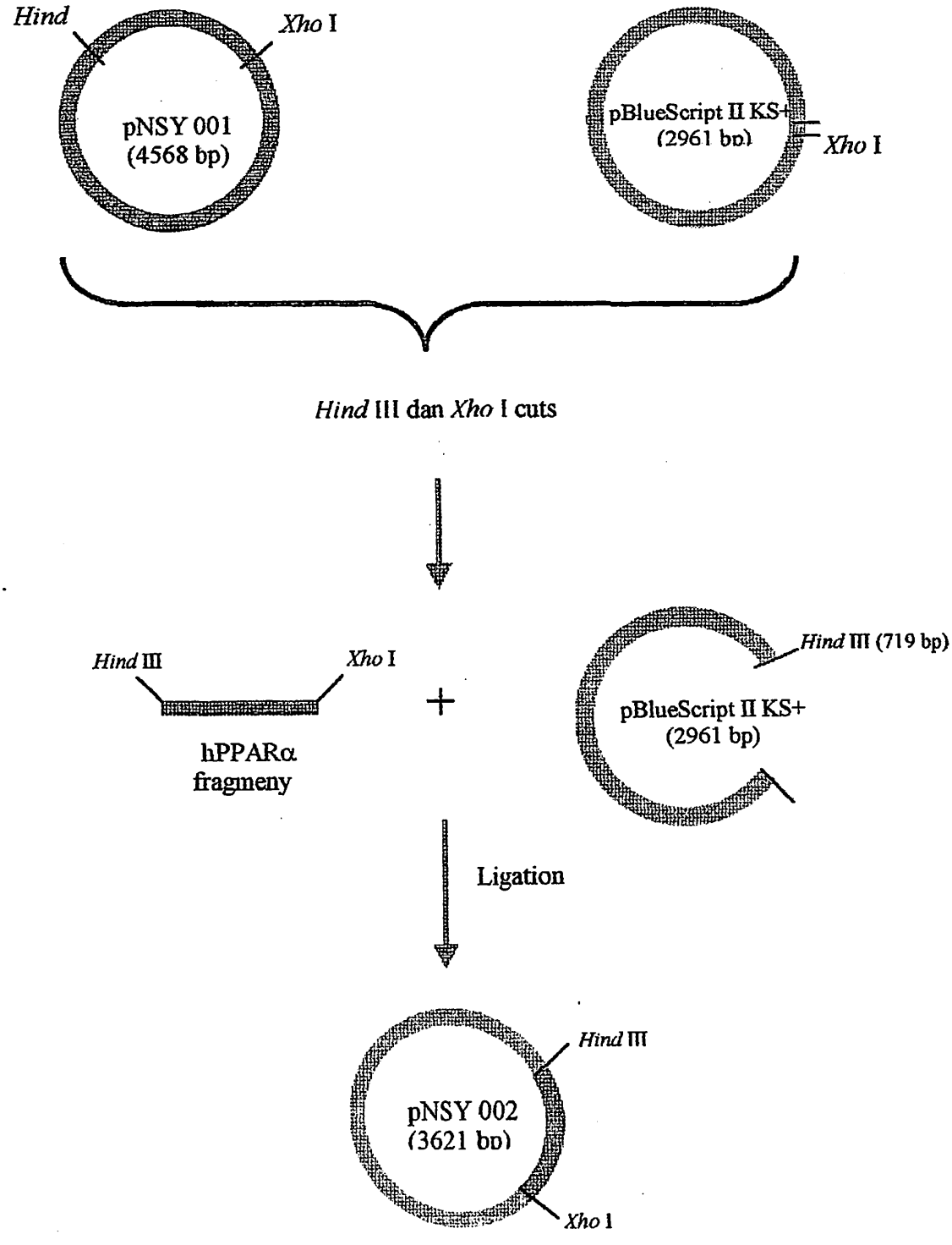


Figure 1: Development of the recombinant plasmid, pNSY 002

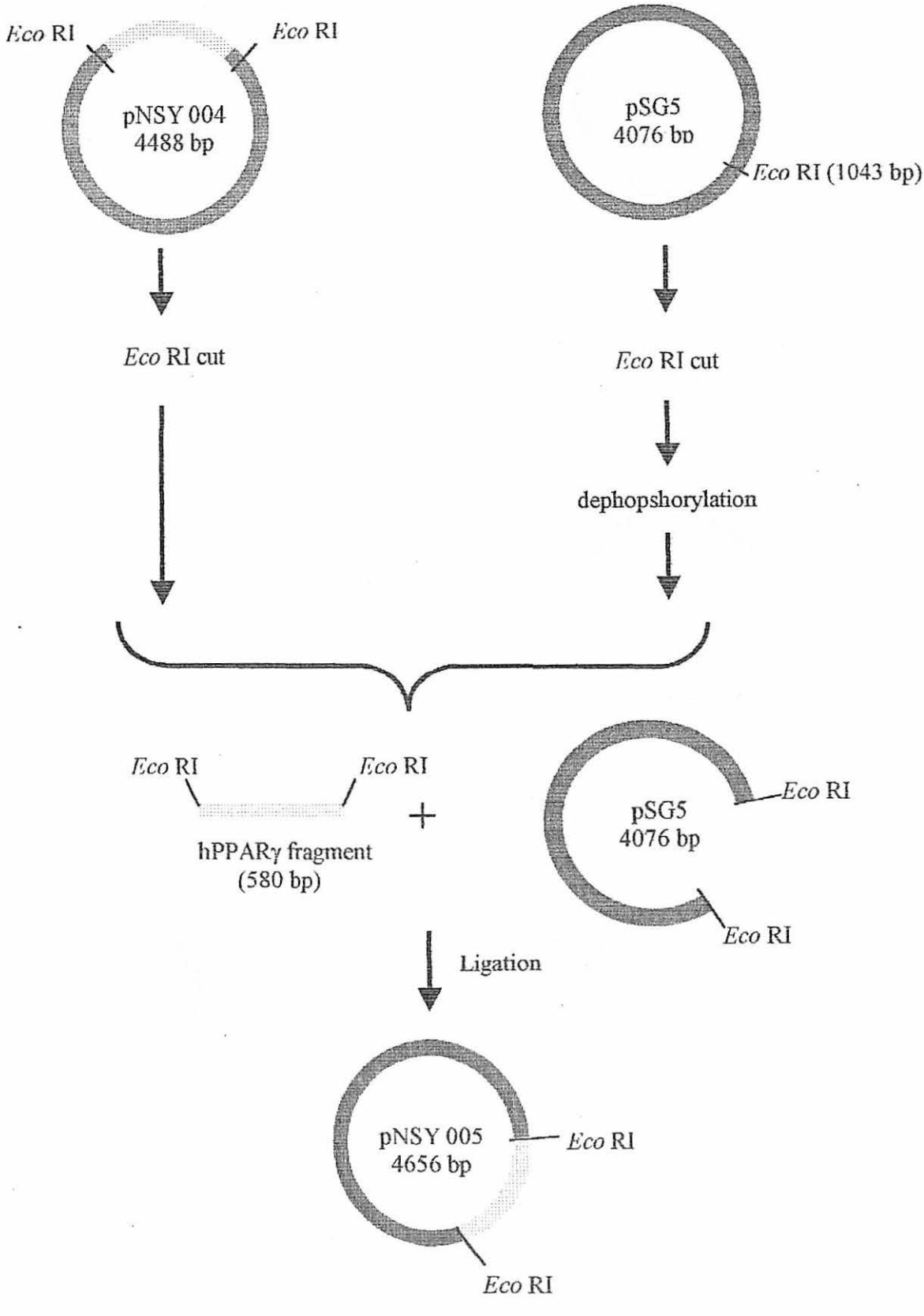


Figure 2: Development of the recombinant plasmid, pNSY 005

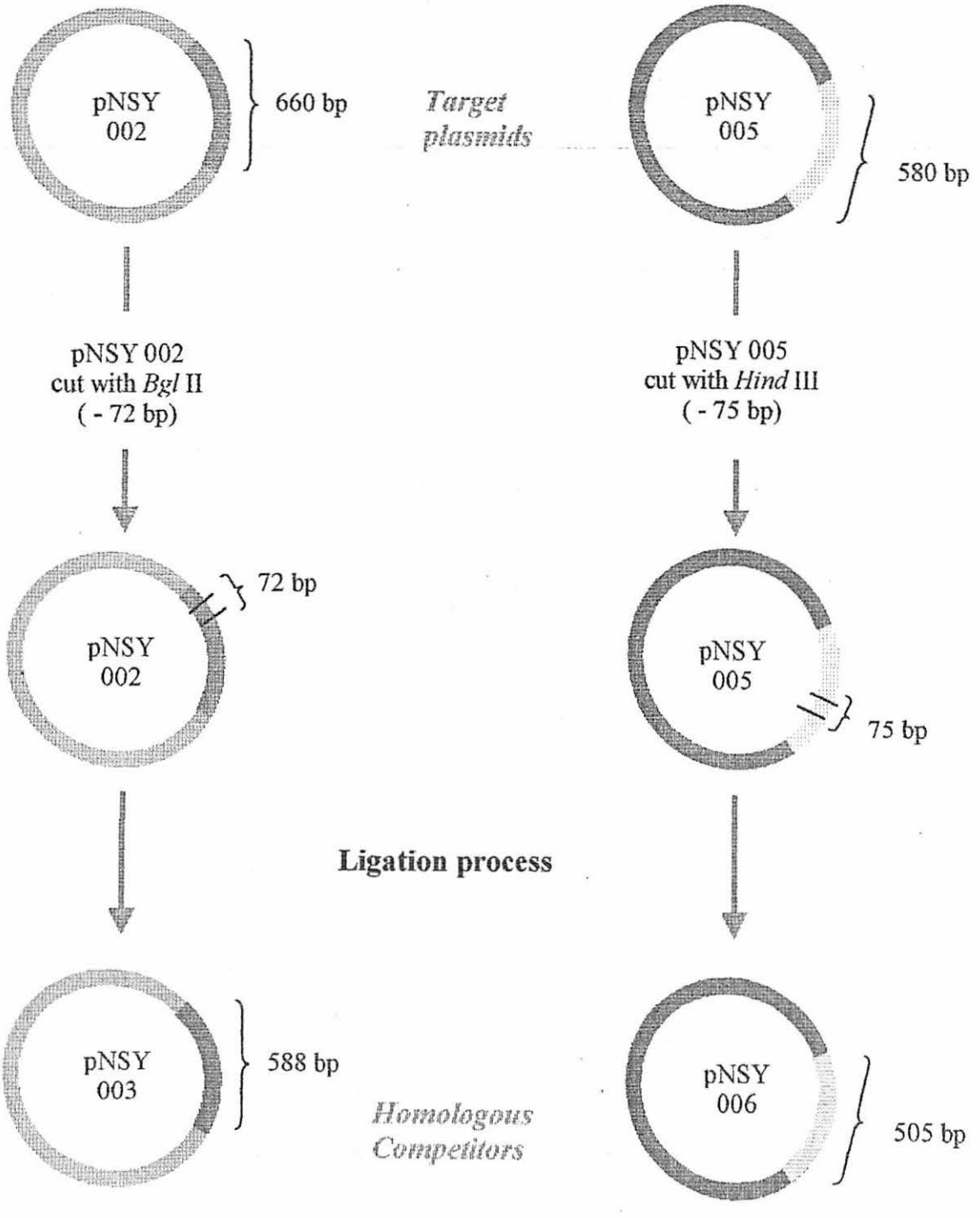


Figure 2: Development of the homologous competitors, pNSY 003 and pNSY 006

Results

All experiments were carried out using the 10-fold standard series to determine the approximate concentration range of the target. Following this, a 2-fold series was used for more accurate determination of the target concentration. In the mock system, we determined the concentration of target by visual or densitometric analyses of the gel electrophoretic bands. In the visual method, when the concentration of target band approximates that of the standard, the intensities of the bands are approximately equal.

An accurate determination of the target can also be performed using densitometry. Figures 4 and 5 show examples of the electrophoreses results following competitive PCR. In Figure 4, gel photographs show results of the competitive PCRs carried out between pNSY 002 and its homologous competitor, pNSY 003, at 10-fold (top) and 2-fold (bottom) serial dilutions of the competitor to determine the concentration of the target gene hPPARα. Figure 5 shows the results of the competitive PCRs between pNSY 005 and the homologous competitor, pNSY 006, for the amplification of hPPARγ. Reactions were also performed with 10-fold (top) and 2-fold (bottom) serial dilutions of the competitor.

Based on the competitive PCRs with the 2-fold dilutions of the competitor, the concentration of hPPARα (Figure 4) and hPPARγ (Figure 5) were approximated at 1.0 x 10^{-3} pmol/μl by the visual method. An accurate detrmination can be performed by measuring the intensities of the electrophoretic bands using densitometry and the graphs plotted as shown in Figure 6. Based on these graphs and taking into consideration the size difference between the target and the competitor, the concentrations of hPPARα and hPPARγ genes were calculated to be at 0.903 x 10^{-3} pmol/μl and 1.135 x 10^{-3} pmol/μl, respectively.

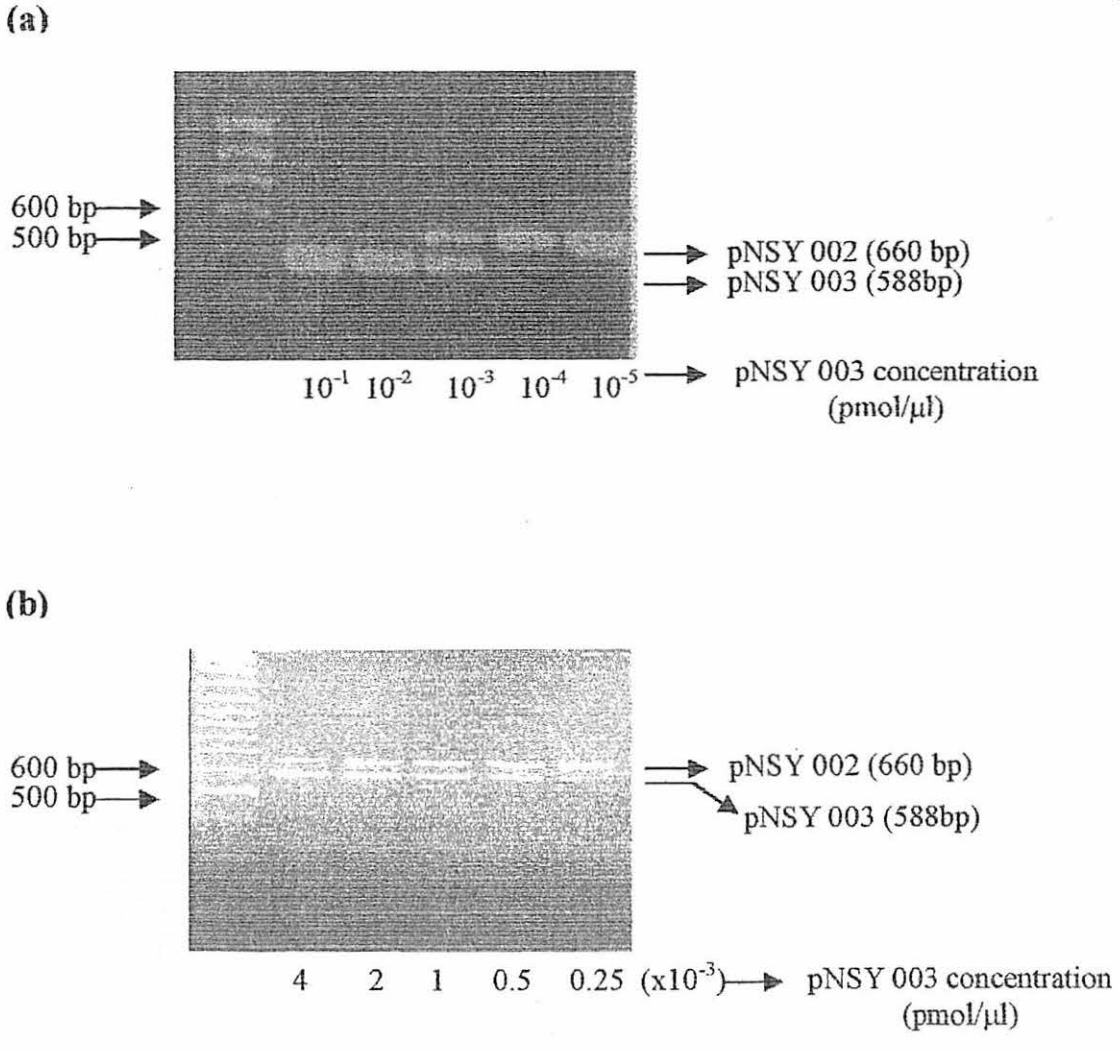


Figure 4: Competitive PCR with hPPARα specific primers using a known amount of pNSY 002 and the hPPARα competitor, pNSY 003, at **(a)**10-fold serial dilutions and **(b)** 2-fold serial dilutions

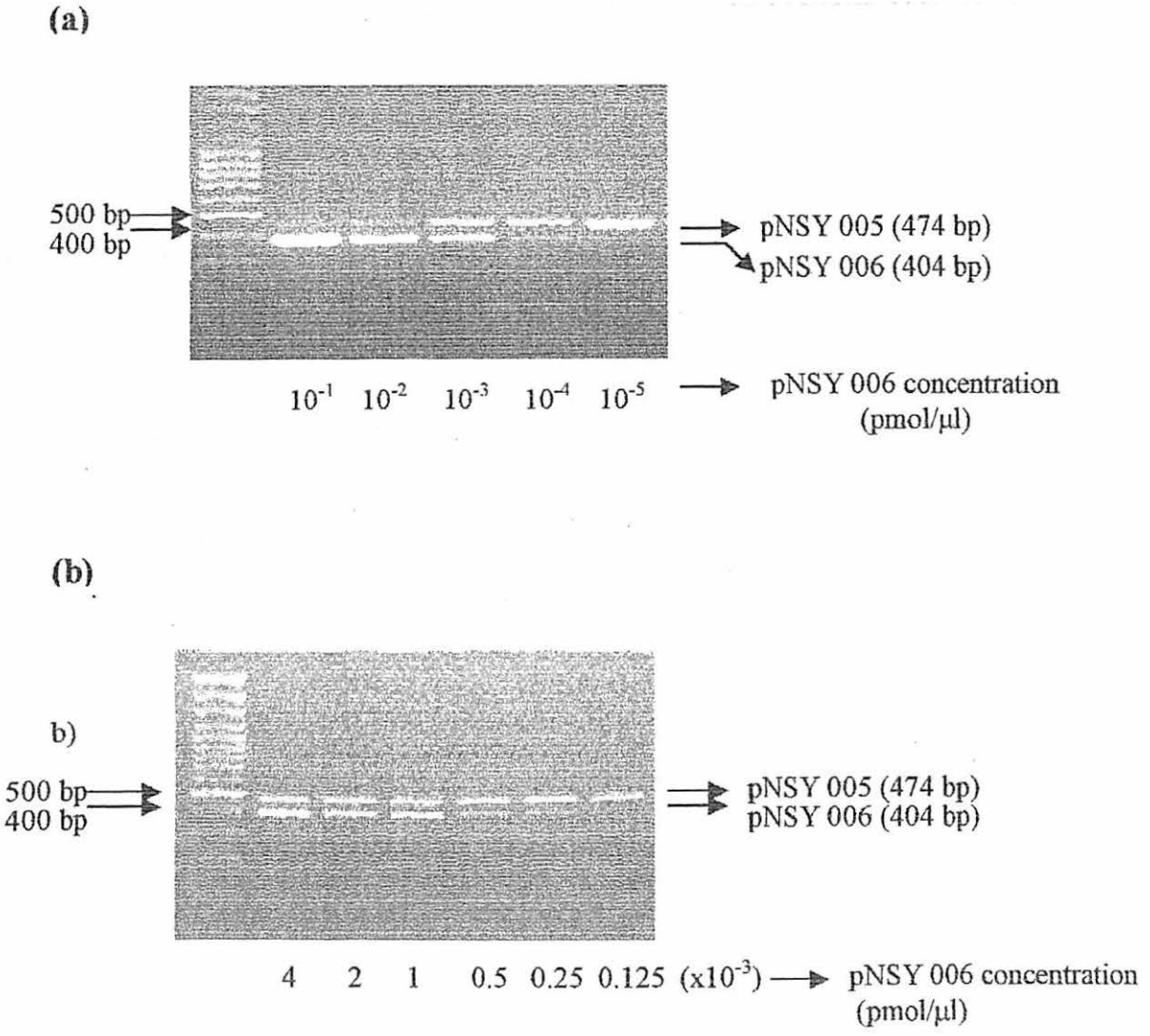
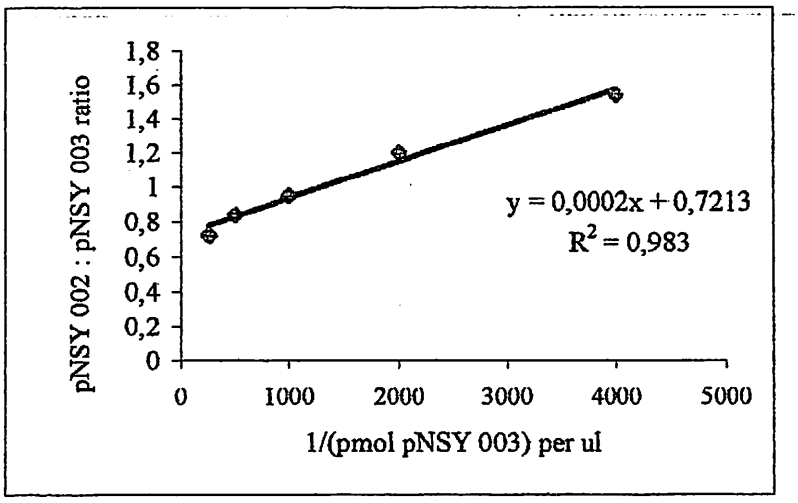


Figure 5: Competitive PCR with hPPARγ specific primers using a known amount of pNSY 005 and the hPPARα competitor, pNSY 006, at **(a)** 10-fold serial dilutions and **(b)** 2-fold serial dilutions

(a)



(b)

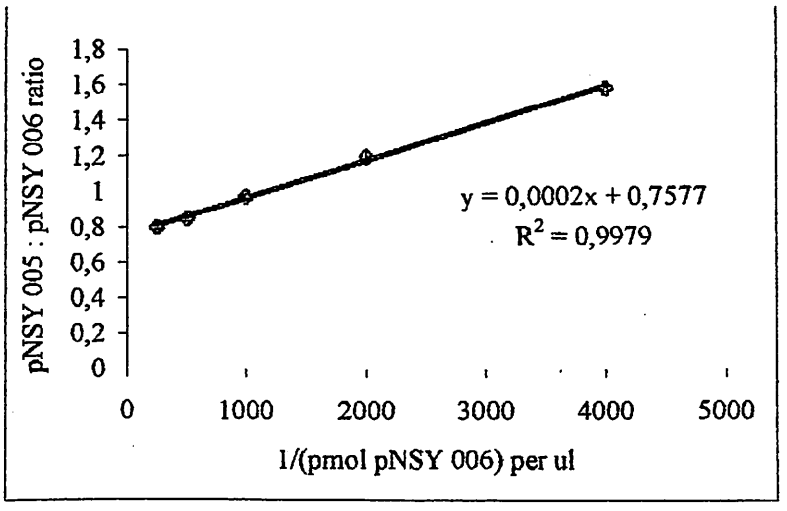


Figure 6 : Competitive PCR graphs plotted following mock experiments (see text) for the calculation of **(a)** hPPARα and **(b)** hPPARγ genes inserted in pNSY 002 and pNSY 005, respectively.

The competitive PCR technique developed was further tested to determine the concentration of target gene in the biological system. An example of an experiment using cDNA of human monocytes is shown in Figure 7. Further experiments were also done using monocytes activated with lipopolysaccharide. It was observed that lipopolysaccharide-activated monocytes resulted in an increase in the PPARγ expression by about 10^3–fold as compared to non-activated monocytes (results not shown).

Conclusion

We have successfully developed a method for gene quantitation using a PCR-based method which utilized a homologous internal standard in a competitive PCR. This method has the advantage over using housekeeping genes as standards since the latter requires different primers and amplification conditions. The current method also has an advantage over using PCR MIMIC which may have different amplification efficiency due to differences in gene sequences between the target and standard used. The homologous standard was constructed from the same target gene with a slightly shorter length to allow for visualization and analysis following competition with the target gene. Mock experiments using known concentrations of targets showed that the method worked successfully. Visual assessment of the target gene revealed concentrations which are similar to the those measured by densitometric analyses. More importantly, the power of quantitation was at the pmole level (10^{-12} mole) or can even be much lower, as we had previously shown with competitive PCR using PCR MIMICS (8), suggesting that this is a very sensitive method for measuring low levels of gene expression. Using cDNA prepared from activated monocytes we were also able to show that it is useful for determining changes in the levels of PPARγ expression in cells expressing the transcription factor. This is consistent with previous findings of an increase in the expression of PPARγ in activated monocytes (10).

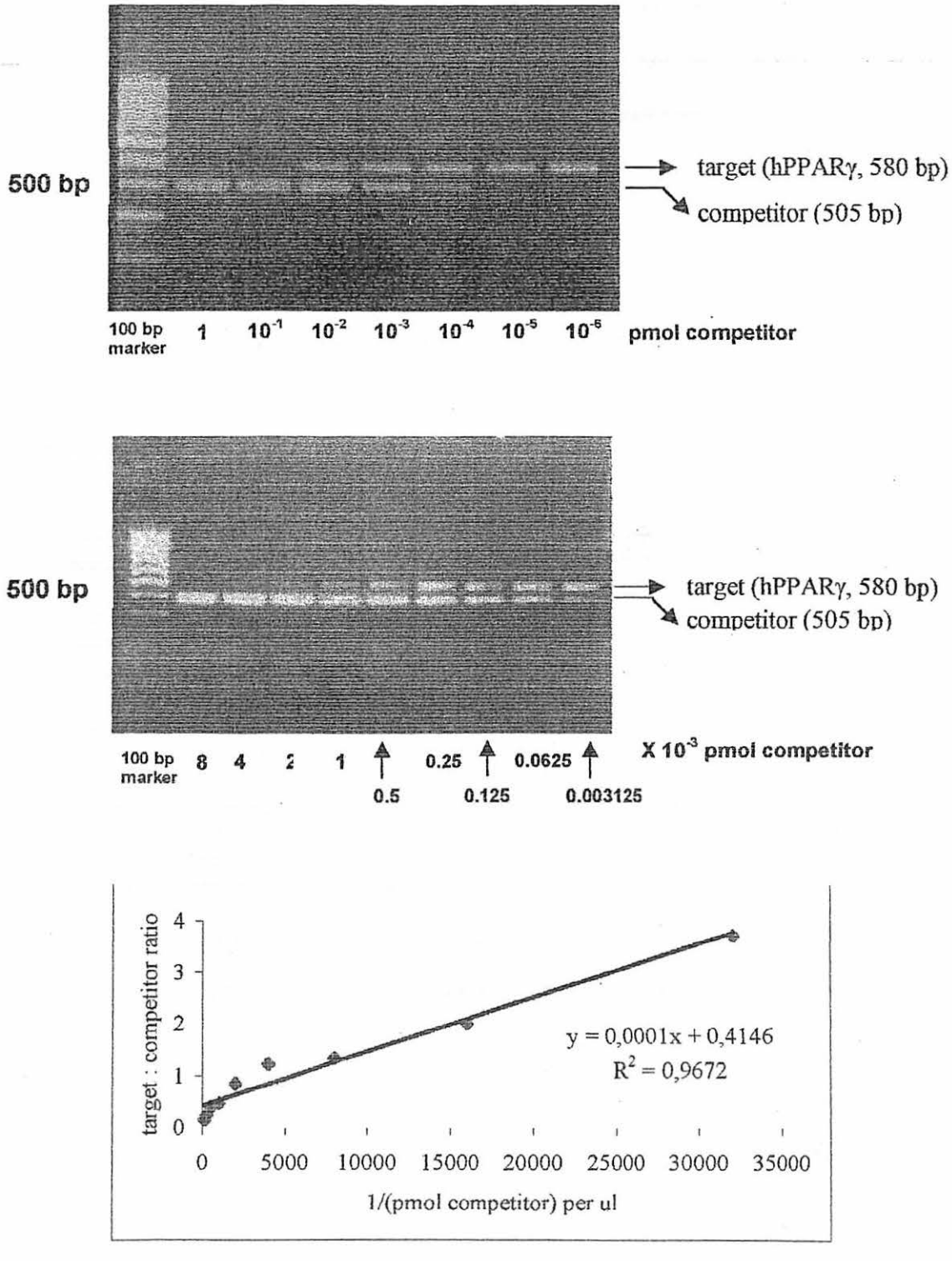


Figure 7: Competitive PCR for the determination of hPPARγ gene expression in human monocytes

In conclusion, we have successfully developed a sensitive and accurate method for the quatitation of gene expression. Furthermore, it is a versatile method whereby approximate concentration levels of gene expression can be discerned visually. Homologous internal standards have been successfully established for the quantitation of hPPARα, hPPARγ, mPPARα and mPPARγ.

Acknowledgements:

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References

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