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# Detection and Analysis of Tupaia Hepatocytes via mAbs Against Tupaia Serum Albumin

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**Abstract**

On the basis of its close phylogenetic relationship with primates, the development of *Tupaia belangeri* as an infection animal model and drug metabolism model could provide a new option for preclinical studies, especially in hepatitis virus research. As a replacement for primary human hepatocytes(PHHs), primary tupaia hepatocytes (PTHs) have been widely used. Similar to human serum albumin, tupaia serum albumin(TSA) is the most common liver synthesis protein and is an important biomarker for PTHs and liver function. However, no detection or quantitative method for TSA has been reported.

In this study, mouse monoclonal antibodies (mAbs) 4G5 and 9H3 against TSA were developed to recognize PTHs, and they did not show cross-reactivity with serum albumin from common experimental animals, such as the mouse, rat, cow, rabbit, goat, monkey, and chicken. The two mAbs also exhibited good performance in fluorescence activated cell sorting(FACS) analysis and immunofluorescence(IF) detection of PTHs. A chemiluminescent enzyme immune assay method using the two mAbs, with a linear range from 96.89 pg/ml to 49609.38 pg/ml, was developed for the quantitative detection of TSA. The mAbs and the CLEIA method provide useful tools for research on TSA and PTHs.

Key words: tupaia serum albumin, primary tupaia hepatocytes, monoclonal antibody

## Introduction

The Asian tree shrew *Tupaia belangeri* (tupaia), a squirrel-like animal phylogenetically related to primates, has been found to be a useful animal model. Genomic analysis has revealed that the tupaia genome is closer to humans than it is to rodents [7]. Histological analysis of tupaia tissues also has been conducted [11, 17]. Because of their similarity in function and structure to primary human hepatocytes(PHHs), primary tupaia hepatocytes (PTHs) have broad uses in research including the study of hepatitis virus infection [8, 13, 16, 21, 27] and prevention [10, 12, 15], virus receptors [9, 18, 23], xenogeneic hepatocyte transplantation [4] and pharmacological testing [26]. Although Tupaia belangeri can be bred in captivity, the lack of tools for their study is a serious limitation [19].

Serum albumin is the dominant protein in the serum, with specific molecular markers indicating the developmental degree and function of hepatocytes in both humans and tupaia. Although tupaia and PTHs have been used frequently in scientific research, the lack of specific detection methods for tupaia serum albumin (TSA) and PTHs has limited their usage. Here, we report two mAbs, 9H3 and 4G5, against TSA, selected to specifically recognize PTHs. Their application in detection methods such as fluorescence activated cell sorting (FACS) analysis, chemiluminescent enzyme immune assay

(CLEIA), immunofluorescence (IF), and western blotting are also described. The mAbs and the CLEIA method could be useful tools in research on the tupaia liver. This study was mainly aimed at promoting the development of a detection method for PTHs and TSA, especially to contribute to the research on tupaia liver chimeric mice.

## Materials and Methods

### Ethics statement

All animal experiments were conducted in accordance with the guidelines of the Xiamen University Institutional Committee for the Care and Use of Laboratory Animals and were approved by the Xiamen University Laboratory Animal Management Ethics Committee.

### Animals and serum

BALB/c mice (purchased from Shanghai SLAC laboratory Animal Co., Ltd.) and Chinese tupaia (purchased from Kunming Institute of Zoology, Chinese Academy of Sciences) were housed in the animal facility at the Xiamen University Laboratory Animal Center. Sera from the tupaia, mouse, rat, cow, rabbit, goat, monkey, and chicken were also provided by the Xiamen University Laboratory Animal Center. Human serum was provided by the Blood Center of Xiamen (Fujian, PR China).

### Hepatocyte collection

Primary human hepatocytes (PHHs; catalog no. CC-2591S) were provided by Lonza (Walkersville, MD, USA). PTHs were isolated by a collagenase

perfusion method from adult male tupaia [13]. All the hepatocytes could be cryopreserved using a programmed cooling device and could be resuscitated with over 90% activity.

**Purification of TSA**

Tupaia serum was diluted and purified with nProtein A Sepharose 4 Fast Flow and Capto DEAE on an ÄKTA™ purifier Box-900 to obtain relatively pure TSA. The concentration of TSA protein was detected and calculated by an Ultrospec 2100 pro. (All instruments and supplies were provided by GE Healthcare Life sciences.)

**Antibodies**

Five female BALB/c mice were immunized subcutaneously with purified TSA emulsified in Freund's complete adjuvant. Two booster doses of the same 50% emulsion with Freund's incomplete adjuvant were delivered to the mice at two-week intervals. The activity of the immune serum with TSA was tested. The highest value was selected to strengthen immunity in the spleen and the construction of the monoclonal antibody cell lines. The mAb 5D2 against human serum albumin (HSA) was introduced in previous research [25]. The mouse IgG (H+L) polyclonal secondary antibody for IF and FACS was Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) (catalog no. A-11001, Invitrogen).

**FACS analysis of mAb binding to hepatocytes**

PHHs and PTHs were treated with 0.1% Triton X-100 in PBS for 3 min and incubated with mAbs 9H3, 4G5, and 5D2 (25℃, 1 h) and washed five times with PBS. The hepatocytes were then incubated with a FITC-labeled rat anti-mouse polyclonal antibody (25℃, 0.5 h) and washed five times with PBS. All the cells were selected under the same conditions. Flow data were acquired on a BD FACSAria™ III (BD Biosciences, San Jose, CA, USA), and data analysis was performed using the BD FACSDiva 5.0.1 software (BD Biosciences, San Jose, CA, USA).

**CLEIA**

CLEIA for HSA is a method previously described in the literature [25]. CLEIA for TSA was performed by the same procedure.

## Results

### 1. High species specificity of mAbs 4G5 and 9H3

Two mice were immunized with TSA purified from the serum of adult tupaia. Two preparations of mAbs 4G5 and 9H3 against TSA were obtained from twelve mAbs for their high affinity. The mAbs 4G5 and 9H3 were labeled with HRP. To determine the species specificity of the mAbs, western blotting was performed to determine whether the mAbs 4G5 and 9H3 exhibit cross-reactivity with serum from humans or seven other commonly used laboratory animals including the mouse, rat, cow, rabbit, goat, monkey, and chicken. The mAbs 4G5

and 9H3 clearly exhibited a reaction only with tupaia serum and TSA among all the tested samples. The two mAbs were used to detect serum from different species coated on plates, and the CLEIA results confirmed that both mAbs only exhibit a special reaction with tupaia serum (Fig. 1A, B, C).

**2. Application of mAbs 4G5 and 9H3 in the detection of PTHs**

The mAbs 4G5 and 9H3 were applied to the detection of PTHs. The results of immunofluorescence detection showed that mAbs 4G5 and 9H3 could distinguish PTHs from PHHs cultured in vitro. The antibody 5D2 against HSA could only recognize PHHs as a control (Fig. 2A). The two mAbs could also recognize PTHs in FACS analysis. Approximately 97.9% of 20,000 PTHs were detected with a positive signal by 9H3, and 96.3% were detected by 4G5, while only 0.4% were detected by the control antibody 5D2 against HSA. 0.03% of 20000 PHHs were detected with a positive signal by both 9H3 and 4G5 as a control, while 97.2% of PHHs were detected by the control antibody 5D2 (Fig. 2B).

**3. CLEIA for the detection of TSA**

For the quantitative detection of TSA in serum or cell culture supernatant, an antibody sandwich CLEIA method using the mAbs 4G5 and 9H3 was developed. The mAb 4G5 acts as the solid-phase antibody, while mAb 9H3 labeled with HRP acts as the detecting

antibody. The linear range of this method was found to be from 96.89 pg/ml to 49609.38 pg/ml via the detection of serially diluted TSA samples (Fig. 3A). As an application, a supernatant of PTHs cultured in vitro was detected using this method: the level of TSA increased with the duration of culture of PTHs, while the control group (PHHs) consistently exhibited negative results for TSA (Fig. 3B). Serum from several adult tupaia older than 6 months and juvenile tupaia approximately 1 month old were tested, and the TSA level showed significant differences between the two groups (Fig. 3C). The TSA level of adult tupaia was always above 20 mg/ml, while in juvenile tupaia, the level was only approximately 5 mg/ml.

## Discussion

Tupaia have been used in scientific research for decades [6] and in the future will probably play more important roles in research fields such as virus infection [5], liver metabolism, regeneration, and disease [22]. In the past 20 years, tupaia and PTHs have been widely used in research on HBV [12, 20, 21] and hepatitis C virus(HCV) [18, 27], especially in the discovery of HBV receptor sodium taurocholate cotransporting polypeptide [23] (NTCP) and the prevention of HBV infection [2, 10, 15]. According to current reports, there are no strains of tupaia, even though many of the tupaia used in research were captured in the wild. The lack of

standard animal strains may result in instability and large individual differences, which limit the widespread adoption of tupaia models. Meanwhile, methods to recognize PTHs or to detect the metabolic function of the tupaia liver are urgently needed for more in-depth study.

In this context, mAbs against TSA would have utility in recognizing and locating PTHs. Furthermore, the CLEIA method for TSA will help in analysis of the liver metabolism of tupaia through detection of the TSA level in the serum. To our knowledge, no similar method has been reported. In the current study, we successfully raised two mAbs against TSA with high species specificity. Both could recognize PTHs cultured in vitro or in suspension. The CLEIA method has a relatively wide linear range for the detection of TSA and thus would provide a useful tool to determine the state of PTHs cultured in vitro or xenografted in vivo, because TSA is one of the most abundant liver proteins and an important biomarker for PTHs and for liver function.

In recent years, advanced chimeric liver mouse models have been developed for liver regeneration and viral infection [1, 3, 14, 24]. PTHs have been transplanted into uPA/*Rag2*$^{-/-}$ mice to build a chimeric liver mouse model for research on HBV and woolly monkey-HBV infection as well as other applications [4]. By detecting the TSA level in the serum of these chimeric liver mice, the chimeric rate of PTHs could be calculated rapidly and easily. Furthermore, the chimeric tupaia liver could be flushed

out and the two mAbs could be used in FACS to analyze the percentage of PTHs directly. Because of the high species specificity of 9H3 and 4G5, the two mAbs may also be used to detect tupaia liver tissues or PTHs in the chimeric liver. The CLEIA method also helps in analyze of the differences between different groups of tupaia at the level of TSA, which indicates the developmental degree and function of the tupaia liver.

**Acknowledgments**

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**Authors Contributions Statement**

C.T. and X.N.S. supervised the design of the research and revised the manuscript. L.X. and Y.L.Z. carried out all the experimental work, analyzed the data, and drafted the manuscript. Y.Q., Z.Y.L., H.W.H.，W.T.Y., and W.K., participated in parts of the experimental work. L.P.G. and S.J.W. participated in the study design and the review of the manuscript.

All authors read and approved the final manuscript.

## Competing financial interests

The authors declare that they have no competing financial interests.

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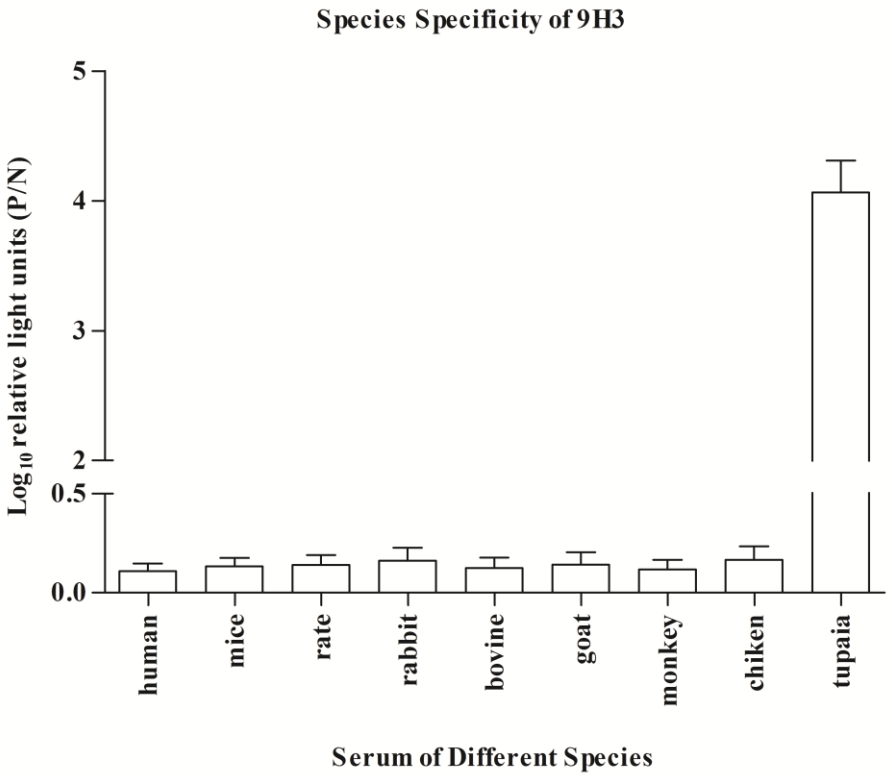
## Figure legends

**Fig. 1 Species specificity of mAbs 9H3 and 4G5 against tupaia serum.** **(**A, B) 9H3-HRP and 4G5-HRP react with serum of the mouse, rat, cow, rabbit, goat, monkey, chicken, and tupaia incubated in a 96-well irradiated plate. (C) Western-blotting assay of 9H3-HRP and 4G5-HRP with sera from different species (bars 1-9: mouse, rat, cow, rabbit, goat, monkey, chicken, and tupaia).

**Fig. 2 Species specificity of mAbs 9H3 and 4G5 against PTH.** (A) The mAbs 9H3, 4G5, and 5D2 incubated with PTHs and PHHs cultured separately in vitro with 6 wells plates, Alexa Fluor® 488 secondary antibody indicating TSA or HSA in the hepatocytes. DAPI staining (blue) marks the nuclei (Plan Apo 63×lens, Zeiss Axio Imager 2 microscope). (B) The mAbs 9H3, 4G5, and 5D2 incubated with PTHs and PHHs suspensions individually and with tubes for FACS, Alexa Fluor® 488 secondary antibody indicating TSA or HSA in the cell population. A total of 20000 cells were analyzed, and the percentage of FITC-positive cells

was calculated.

**Fig. 3 CLEIA method for detection of TSA.** (A) Range and linearity of the CLEIA method for TSA. A sample of TSA with a concentration of 25.40 mg/mL was double diluted 21 times to 12.11 μg/ml. The diluted samples were detected using the CLEIA method for TSA. The method exhibited stable linearity ranging from 96.89 pg/ml to 49609.38 pg/ml ($R^2$=0.9751). (B) Detection of the TSA level in the supernatant of PTHs/PHHs from different tupaia cultured in vitro: PHHs and PTHs from each tupaia were cultured individually in 4 different 10 cm plates, and the TSA level increased after the hepatocytes were cultured. (C) Detection of the TSA level in serum from 3 adult and 3 juvenile tupaia. Each tupaia had serum collected at 4 different time points. The groups of adult and juvenile tupaia exhibited significant differences ($P< 0.0001$).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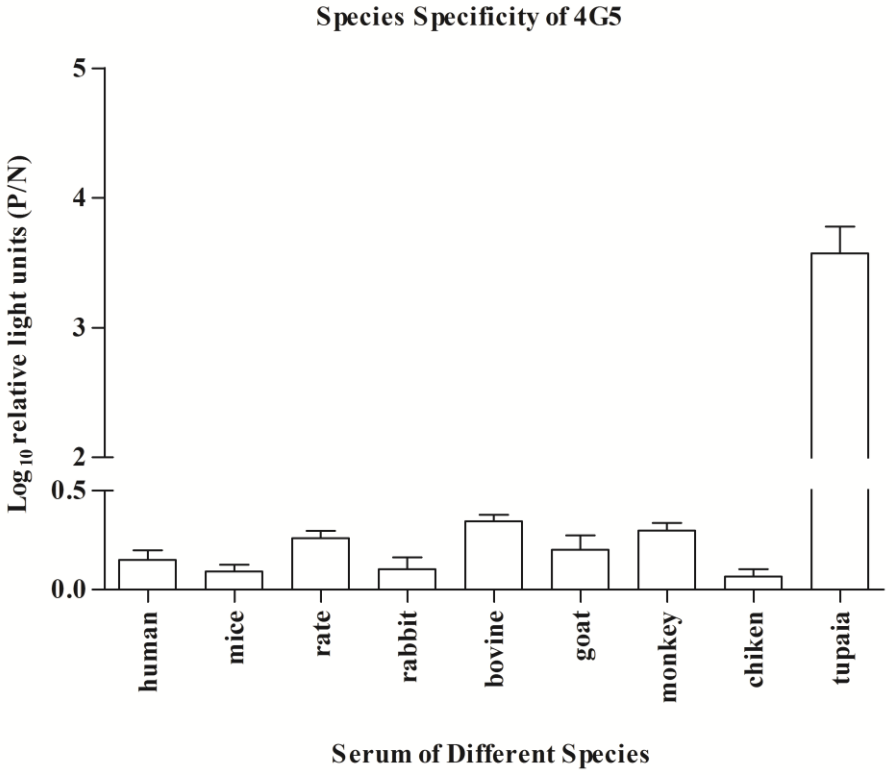
Serum of Different Species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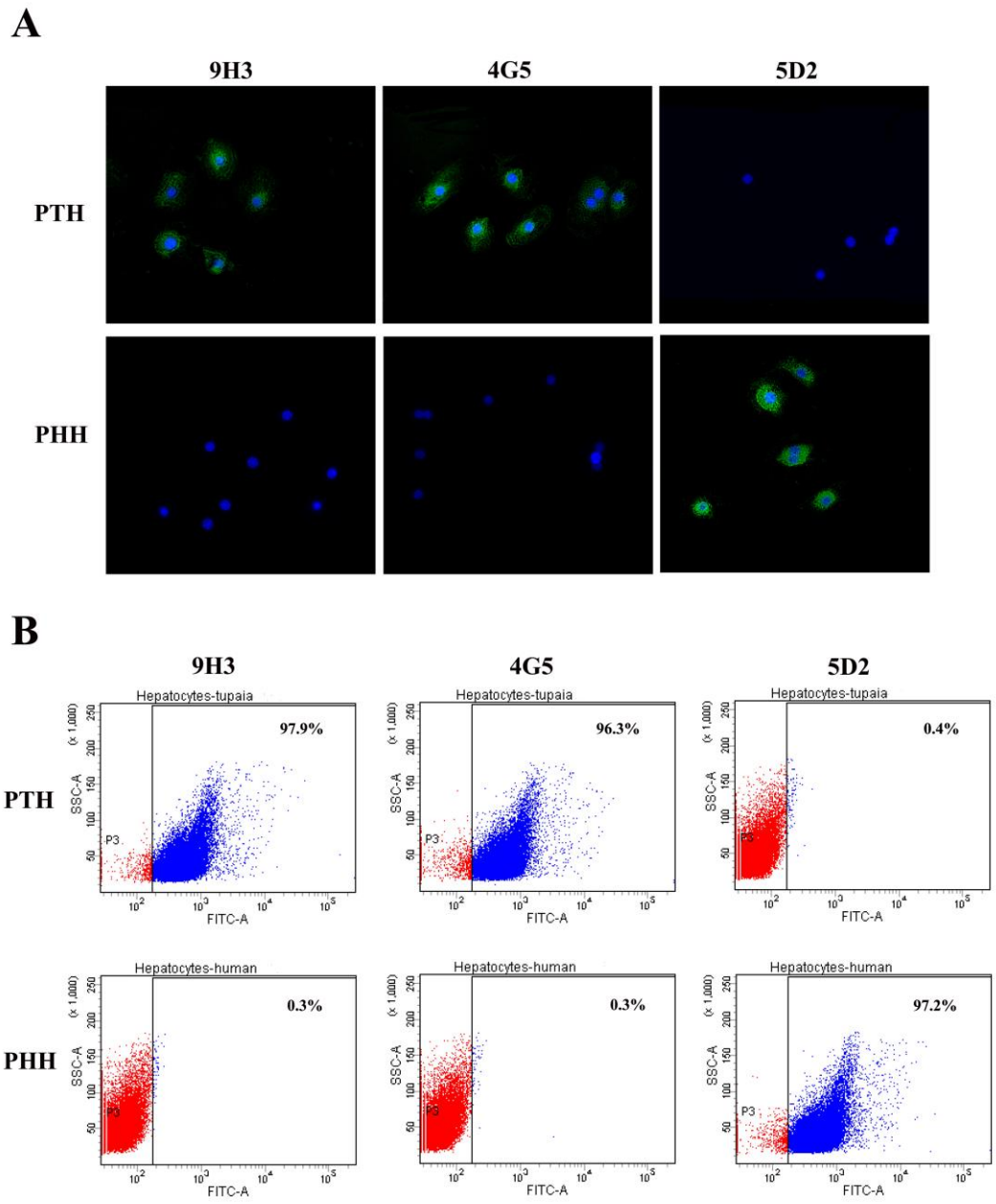
Serum of Different Species

Fig. 1


A
9H3
4G5
5D2
PTH
PHH
B
9H3
4G5
5D2
PTH
PHH
Hepatocytes-tupaia
Hepatocytes-human
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96.3%
0.4%
0.3%
0.3%
97.2%
SSC-A
FITC-A
P3

Fig. 2

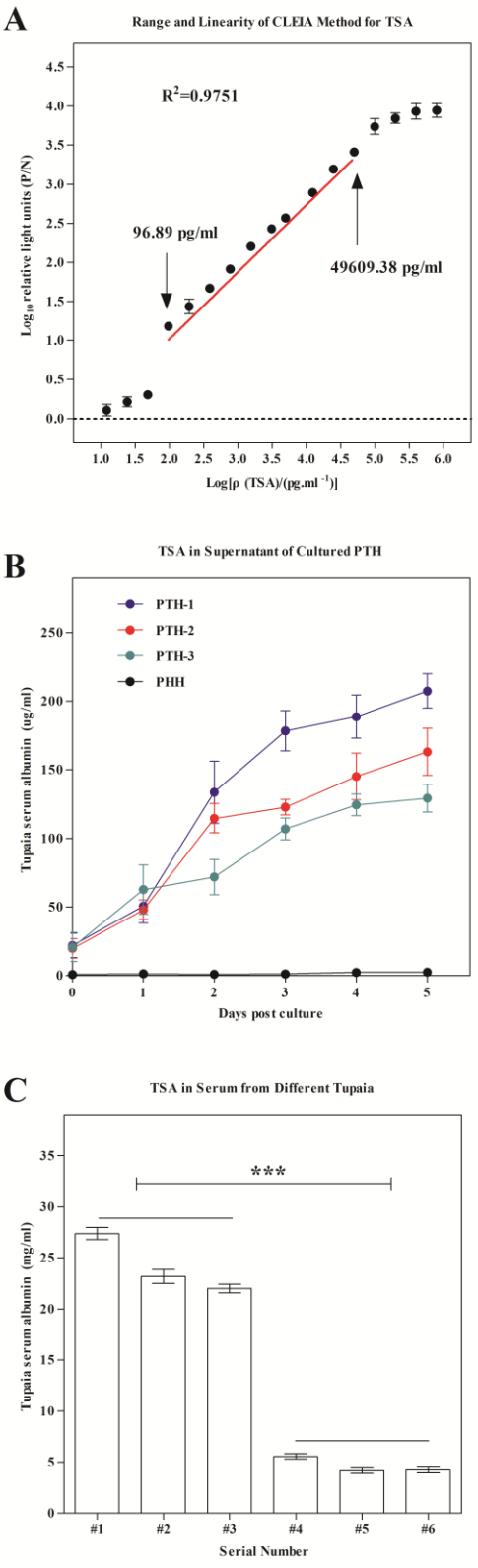
A
Range and Linearity of CLEIA Method for TSA
R²=0.9751
96.89 pg/ml
49609.38 pg/ml
Log10 relative light units (P/N)
Log[ρ (TSA)/(pg.ml⁻¹)]
B
TSA in Supernatant of Cultured PTH
PTH-1
PTH-2
PTH-3
PHH
Tupaia serum albumin (ug/ml)
Days post culture
C
TSA in Serum from Different Tupaia
***
Tupaia serum albumin (mg/ml)
#1
#2
#3
#4
#5
#6
Serial Number

Fig. 3