A new reliable and sensitive nested PCR assay based on the human SRY gene for detection of interspecific chimeras

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Abstract: Although interspecific chimeras can be identified using laborious techniques, more accurate and rapid detectable methods need to be established. The present study develops a nested polymerase chain reaction (nPCR) assay to detect human–mouse chimeras. A set of previously validated outer primers, specific to the human SRY gene, was used for conventional one-step PCR, while the inner primers for nPCR were designed. The specificities of PCR and nPCR assays were examined; both primer sets yielded PCR amplification products from male human epidermis-derived mesenchymal stem cell-like pluripotent cell DNA but no amplification products from negative control DNA. The sensitivity of this nPCR was determined using mixed DNA. Measurable amplification of SRY transcripts was a male human to female mouse DNA ratio of 1:10,000. We then tested the nPCR assay on tissues from female mouse chimeras. The nPCR products were selected randomly for sequencing and positive samples were further analyzed by fluorescence in situ hybridization (FISH) using specific probes for the human SRY gene and by immunofluorescence staining for species-specific markers. There was 100% concordance among nPCR, FISH, and immunofluorescence results, and the nPCR product sequences were consistent with the human SRY gene. Taken together, we developed a new, highly specific, sensitive, and reliable method to detect human–mouse chimeras.

Key words: Interspecific chimeras, nPCR, Y chromosome, SRY gene, cell differentiation, stem cell, regenerative cells

1. Introduction
Chimeric animals provide specific in vivo environments for cell differentiation and are widely used to evaluate cell lineage potential and to define growth factors and substrates required for specific developmental processes. Chimeras were first exploited to study early embryonic development, but can also be used to analyze organogenesis, postnatal maturation, and mature physiological function (Tam and Rossant, 2003). Chimeras are typically generated by transplanting fetal or adult tissues from one individual to another and by grafting embryonic stem cells or their differentiated products into another individual (Behringer, 2007).

Several analysis techniques have been developed to determine the origins of specific cells and tissues within chimeras. Polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) are useful to detect the genes of donor cells (Duffield and Bonventre, 2005; Huang et al., 2008). Strategies for detecting proteins from donor cells include enzyme-linked immunosorbent assays (ELISA), immunofluorescence, green fluorescent protein (GFP) tagging, and mass spectroscopy (Najimi et al., 2007; Ramírez et al., 2009; Kobayashi et al., 2010). Using immunohistochemistry, we can distinguish donor cells from recipient cells based on morphology (Meuleman et al., 2005). It is relatively easy to obtain allogeneic chimeras with abundant surviving donor cells and to monitor engraftment. However, chimeras created with few donor cells require greater sensitivity in their methods. Various PCR techniques have been developed in the last two decades to evaluate chimeras, including nested PCR (nPCR), real-time PCR (RT-PCR), and short tandem repeats PCR (STR-PCR).

Nested PCR involves a two-step PCR reaction. In the first step, PCR amplification is performed with primers specific for a given target gene. Then the amplified PCR products are subjected to a second amplification using nested or inner primers. nPCR has higher specificity and sensitivity than conventional PCR and thus may be superior for detecting donor cells present in low numbers.
in the host tissue. Indeed, highly specific and sensitive nPCR protocols have been developed for both clinical diagnosis and experimental studies (Frenoy et al., 1994).

In the present study, we developed a new nPCR method using primers designed for the human sex-determining Y (SRY) gene located on the Y chromosome. The specificity and sensitivity of the nPCR method were assessed by generating standard PCR curves from known mixtures of male human epidermis-derived mesenchymal stem cell-like pluripotent cell (hEMSCPC) DNA (Huang et al., 2013) and human female blood or female chimeric mouse DNA. The utility of this nPCR technique was evaluated in multiple chimeric tissues by comparing results to those obtained from FISH and immunofluorescence staining. The high concordance among these different techniques indicated that this new nPCR technique is a convenient, sensitive, and selective method for detecting the origins of cells and tissues within chimeras.

2. Materials and methods

2.1. Ethics statement

This study was performed with the approval (No.[2008] 30) of the Medical Ethics Committee of the Zhongshan Ophthalmic Center, Sun Yat-Sen University, Guangzhou, China. The male hEMSCPCs used in this study were described in a previous publication (Huang et al., 2013). They were approved for use in the present study by the Medical Ethics Committee of the Zhongshan Ophthalmic Center, Sun Yat-Sen University (No.[2008] 30). The human female blood samples used in this study were donated by a healthy individual (n = 1). ‘Healthy’ was defined as having normal blood and urine lab tests, normal liver and lung function, no history of genetic disease, and absence of infectious diseases. Written informed consent was provided by the participants. All procedures involving animals were approved by the Animal Ethics Committee of the Zhongshan Ophthalmic Center, Sun Yat-Sen University (Animal Welfare Assurance No. 2010-024).

2.2. Primer design

The nucleotide sequence for the human SRY gene (NG_011751.1, GenBank) was used to design primers for conventional one-step PCR: F1, 5’-CAGTGTGAAACCGGAGAAAACAGT -3’ and R1, 5’-CTTCCGACGAGGTGATCTTATA-3’ (Semerci et al., 2007). The predicted PCR product was 270 bp. The nPCR primers, designed using Primer 5 software, were F2, 5’-TGTATTTCTGTCCTCTGGAAGAATGG-3’ and R2, 5’-GAAACGGGAGAAAACGTTAAGGCAAACGT -3’. The predicted PCR product, following the two-step nPCR, was 212 bp. The GAPDH gene was amplified as an internal control using primers F , 5’-TCACTCAAGATTGTCAGCAA-3’ and R, 5’-AGATCCACGACGGACACATT-3’. The PCR and nested PCR products were separated on 1.5% agarose gels and visualized under a UV light using Goldview DNA stain (Guangzhou Geneshun Biotech Ltd., China).

2.3. DNA extraction

Genomic DNA samples from female human blood, male hEMSCPCs, and blood, brain, heart, lung, liver, kidney, and spleen tissues from female chimeric mice (created by grafting male hEMSCPCs into mouse blastocysts) were extracted using the Biomiga EZgene Tissue gDNA Miniprep Kit. The DNA samples were stored at –20 °C until used for conventional PCR or nPCR.

2.4. Tests for specificity and sensitivity of the nPCR technique

To evaluate the specificity of the nPCR method, we used male hEMSCPCs as the positive control DNA source and female human and mouse blood as the source of negative control DNA. To evaluate the sensitivity of nPCR, results were compared to conventional one-step PCR using standard mixtures of male hEMSCPC DNA and DNA from the blood of female mice. The separate DNA concentrations were determined by ultraviolet spectrophotometry with mixture ratios of 1:1, 1:10, 1:100, 1:1000, 1:2000, and 1:10,000. Total weight for both species of DNA samples was 30 ng for the lowest 4 ratios, 100 ng for 1:2000, and 500 ng for the 1:10,000 mixtures (Wang et al., 2002).

2.5. PCR and nested PCR amplification

The PCR reactions were performed in a 25-µL volume containing 12.5 µL of 2X PCR mix, 18.5 µL of ddH2O, 1 µL of each primer at 10 pmol/µL, and 2 µL of each DNA template. The thermocycle conditions were a single 10-min step at 95 °C to activate the DNA polymerase and then 35 cycles of 30 s of denaturation at 95 °C, 30 s of annealing at 65 °C, and 30 s of extension at 72 °C, followed by a final 10-min extension at 72 °C. Reactions were performed in a professional standard Gradient Thermocycler (Biometra GmbH, Germany). The conventional one-step PCR amplification was performed using primers F1 and R1. The amplified PCR products (0.5 µL) were used for the subsequent nPCR with 20 µL of ddH2O and 34 cycles. The thermocycle protocol for GAPDH followed that of SRY, except only 27 cycles were used.

The PCR and nested PCR products were separated on 1.5% agarose gels and visualized under a UV light using Goldview DNA stain (Guangzhou Geneshun Biotech Ltd., China).

2.6. Construction of chimeric mice

The hEMSCPCs, frozen in liquid N2 at P10–P13 in 10% DMSO, were thawed in a 35–37 °C water bath, suspended in DMEM containing 10% FBS, and centrifuged at 1200 rpm for 5 min. The supernatant was removed and the cell pellet was resuspended in 0.5 mL of serum-free DMEM, incubated at 6–8 °C for 10 min, and then injected into mouse blastocysts under a light microscope. Each mouse...
blastocoel was injected with 6–8 cells. Injected mouse embryos were incubated overnight at 36.8 ± 0.2 °C under 5% CO₂ and 100% humidity. On the second day, well-developed embryos were transferred into the uteruses of 2.5-day pseudopregnant female mice. In 18 ± 1 days, pups were born. Among the pups surviving for more than 1 month, only females were used for the study.

2.7. FISH and immunofluorescence staining of chimera blood

The antibodies used for immunofluorescence staining exhibited no cross-reactions in wild-type female mice (data not shown). A 200-µL volume of anticoagulated blood was obtained from female offspring, added to 3 mL of 0.83% NH₄Cl solution precooled at 4 °C, gently mixed by pipetting, and then left to stand at 37 °C for 15 min. Next, 4 mL of PBS was added and mixed by gentle pipetting. The new mixture was centrifuged at 1500 rpm for 5 min and the supernatant was discarded. A 100-µL volume of PBS was added to the centrifuge tube and the mixture was gently pipetted to suspend the cells. All subsequent steps were performed under light shielding. An FITC-labeled antihuman monoclonal antibody against CD3 (Invitrogen, USA; dosage based on the protocol provided by manufacturers) was added to the cell suspension and gently mixed by pipetting. The labeled suspension was incubated at room temperature for 30–40 min as recommended by the reagent manufacturers. The suspension was centrifuged (1800 rpm for 5 min), the supernatant was discarded, and the cells were resuspended in PBS by vortex mixing. Following a second cycle of centrifugation–resuspension in PBS, 1 mL of fixative solution, consisting of pure methanol and glacial acetic acid (3:1), was added drop-wise while the cell suspension was gently blended. The fixed and FITC-labeled cell suspension was then centrifuged at 2000 rpm for 5 min, the supernatant was removed, and the cells were resuspended in 100 µL of the same fixative solution. The solution underwent cytospinning (Shandon, Thermo Electron Corporation, UK) at 2000 rpm with moderate acceleration for 2 min, resulting in the cell smears being air-dried. Smears were soaked in rinsing solution containing 2X SSC plus 0.1% NP-40 preheated at 37 °C for 30 min (prepared based on the protocol provided by Abbott Molecular Inc., USA). Excess solution on the smears was removed and gradient dehydration was performed in an ascending ethanol series (70%, 85%, and 100% at room temperature with 2 min at each concentration), followed by air-drying. The following procedures were performed under light shielding. A 10-µL volume of human-Y specific probe mixture (prepared based on the regimen provided by Abbott Molecular Inc.) was pipetted onto the smears. The labeled smears were then covered with slips, incubated for 6 to 7 min at 73 ± 1 °C for denaturation, and then incubated in a sealed humid container preheated at 42 °C for 16 h for annealing. The cover slips were removed and the smears were rinsed with 2X SSC/0.3% NP-40 solution (prepared according to the protocol provided by Abbott Molecular Inc.) at 73 ± 1 °C for 2 min and then transferred to 2X SSC/0.1% NP-40 solution at room temperature for another 2 min. Excess solution was removed from the smears, which were then air-dried, counterstained with 10 µL of DAPI solution (Abbott Molecular Inc.), and blocked by upturning the smear plates. Stained smears were observed and photographed 30 min later under a laser confocal microscope (Zeiss, Germany) using excitation/emission pairs (in nm) 405/461, 488/525, and 543/588.

2.8. FISH and immunofluorescence staining of chimera tissue

All antibodies used were tested for cross-reactivity on tissue from wild-type female mice (data not shown) before experiments. The antibodies tested included a rabbit antihuman MAP2 monoclonal antibody (Abcam, USA) and FITC-labeled mouse or rabbit antihuman monoclonal antibodies against SP-C, troponin I, CD16, ALB, and VEGF R2 (Invitrogen, USA; Santa Cruz, USA; BD, USA). The MAP2 antibody was visualized using an FITC-conjugated goat antirabbit secondary antibody (SouthernBiotech, USA). Brain, heart, lung, spleen, liver, and kidney tissues were isolated from wild-type and chimeric female mice, embedded in OCT compound (Sakura Finetek, USA), and stored at –20 °C for subsequent PCR, FISH, and immunohistochemical analyses. Tissues that tested positive for the SRY gene by nPCR were sliced at 4 µm and air-dried, and the OCT gel was removed. To improve adhesion of tissue to slides and to adequately spread tissue structures, cytospinning (Shandon, Thermo Electron Corporation) was performed at 2000 rpm with moderate acceleration over 2 min. The following protocols were performed under light shielding. For immunostaining, smeared slices were incubated with one of the primary antibodies listed above at room temperature for 40 min, then rinsed 3 or 4 times with PBS at 6–8 °C. For MAP2 immunostaining, the tissue was incubated in an FITC-conjugated secondary antibody at room temperature for 30 min and rinsed 3 or 4 times with PBS, which had been precooled to 6–8 °C. After incubation with antibodies, tissue slices were air-dried. FISH was performed on the slices that had not been treated with PBS. Finally, stained slices were observed and photographed under a laser confocal microscope (Zeiss, Germany) using the excitation/emission pairs (in nm) 405/461, 488/525, and 543/588.

2.9. DNA sequencing

Positive DNA products from the nPCR specificity test and from tests of chimeric mouse tissue were sequenced by the Beijing Genomics Institute, Beijing, China.
3. Results

3.1. Specificity and sensitivity of the PCR and nPCR methods
Female human blood DNA, female mouse blood DNA, and male hEMSCPC DNA were used as templates for the specificity test. The species-specific SRY primers yielded amplicons of 270 bp using male hEMSCPC DNA as the template, while no amplicons were observed when female human blood DNA or female mouse blood DNA (100 and 500 ng, respectively) were used as the template (Figure 1A). The nested primers amplified a 212-bp inner span of the human SRY gene from hEMSCPC DNA (Figure 1B), whereas no amplicons were observed using the female templates. The amplicons from PCR and nPCR tests were sent to the Beijing Genomics Institute for sequencing and the results confirmed that the sequences were almost 100% identical to the human SRY gene.

In the sensitivity test, genomic DNA from male hEMSCPCs and female mouse blood DNA were mixed at male to female ratios of 1:1, 1:10, 1:100, 1:1000, 1:2000, and 1:10,000. Sample weight was 30 ng for the lowest four ratios, 100 ng for the 1:2000 mixtures, and 500 ng for the 1:10,000 mixtures. For conventional one-step PCR, a 270-bp nucleotide band was detected only at ratios of 1:1, 1:10, and 1:100. The efficiency of the amplification using the 1:10 DNA mixture was very low, and we detected no products at ratios lower than 1:100 (Figure 1A). In contrast, 212-bp PCR products were detected for all ratios following two-step nPCR (Figure 1B).

3.2. Detection of SRY DNA in chimeras
In a previous study, we created interspecific chimeras by grafting male hEMSCPCs into mouse blastocysts. We collected genomic DNA samples from the blood, brain, heart, lungs, liver, spleen, and kidneys of female mice offspring to compare the utility of nPCR to conventional PCR (Figures 2A–2G). The one-step PCR detected human DNA in only 1 of 88 samples (1.14%) (Figure 2C), whereas the nPCR detected human DNA in 14 of 88 samples (15.91%) (Figures 3A–3G). The concordance rate between PCR and nPCR was
Figure 2. Detection of chimeric tissues using PCR. Blood (A), brain (B and C on the left), heart (B and C on the right), lung (D on the left), liver (D on the right), kidney (E–G on the left), and spleen (E–G on the right). In all panels, lane M, marker; lane H₂O, blank control; lane N (negative control), 100 ng of female mouse DNA; lane P (positive control, male hEMSCPC DNA); lane (number), refers to the identification number of the female mouse tested.
Figure 3. Detection of chimeric tissues using nPCR. Blood (A), brain (B and C on the left), heart (B and C on the right), lung (D on the left), liver (D on the right), kidney (E–G on the left), and spleen (E–G on the right). In all panels, lane M, marker; lane H\_2O, blank control; lane N (negative control), 100 ng of female mouse DNA; lane P (positive control, male hEMSCPC DNA); lane (number), refers to the identification number of the female mouse tested.
Sequences of the nPCR products showed that the sequences of the positive sample (A) and heart tissue sample 36 (B) were almost 100% identical to the human SRY gene (Figures 4A and 4B).

3.3. Comparison of results from nPCR, FISH, and immunofluorescence staining for detection of chimeric tissue

To confirm the accuracy and utility of nPCR for the detection of chimeras, we compared the results of nPCR, FISH, and immunohistochecmistry for detection of the human SRY gene in blood, brain, lung, heart, spleen, liver, and kidney tissue samples from female chimeras. First we determined that male hEMSCPCs were positive for SRY, but negative for all other proteins targeted by all other FITC-conjugated antibodies (Figure 5A). No SRY-positive and antibody-positive cells were detected in the blood of wild-type female mice (Figure 5B). Then we analyzed the contribution of hEMSCPCs to the various tissues in female chimeras. Fluorescent imaging indicated that all chimeric tissues shown to be SRY-positive by nPCR were also SRY-positive, as determined by FISH and immunofluorescence. In addition, all SRY-positive tissues expressed several specific human antigens, including CD3 (in blood cells), MAP-2 (in brain cells), SP-C (in lung cells), troponin-1 (in heart cells), CD16 (in spleen cells), ALB (in liver cells), and VEGF R2 (in kidney cells) (Figures 5C–5I). These results indicated that the positive samples detected by nPCR contained cells originating from hEMSCPCs.

4. Discussion

Chimeras can be generated by allografting or xenografting to produce interspecies chimeras. Allograft chimeras are typically generated by grafting homogeneous same-species cells into embryos, fetuses, or even adults (Shaharuddin et al., 2013; Ozaki et al., 2014; Sgambat and Moudgil, 2014). Examples of allograft chimeras as stem cell and organic transplantations in the clinical setting include autologous stem cell, renal, and cornea transplantation. Allografted tissues result in higher donor cell survival due to limited immunologic rejection. If the donor tissue and the recipient are from different species, the immune suppression becomes very strong and the birth rate of interspecific chimeras is low. An immune-deficient mouse and a goat in a preimmune state strain have been used as the host for heterogeneous cell grafts to generate interspecific chimeras with high donor cell and long-term survival (Meuleman and Leroux-Roels, 2008; Zeng et al., 2013). However, it is difficult to generate interspecific chimeras with high donor cell numbers from immunologically normal hosts. This low cell survival rate necessitates tests of high specificity and sensitivity to detect donor tissues.

Fluorescent fusion proteins are a powerful method to detect donor cells and proteins in the tissues and organs of the host (Lippincott-Schwartz et al., 2001; Yang et al., 2005; Giepmans et al., 2006; Shaner et al., 2007; Shcherbo et al., 2007). GFP is one of several proteins that can be fused to a native protein and inserted into the genome to trace and detect donor cells in the host tissue with high specificity.
sensitivity and stability (Eckardt et al., 2008; Kanatsu-Shinohara et al., 2008; Oda et al., 2009). However, several studies have demonstrated the limitations of this method (Liu et al., 1999), as GFP can alter development and mature function, especially when expressed at high levels (Ho et al., 2007; Mawhinney et al., 2011). Moreover, fluorescence microscopy is too time-consuming to screen large numbers of potential chimeras. In a previous study,
secretory proteins from donor cells were measured in chimeras with ELISA and mass spectroscopy (Meuleman et al., 2005), but these methods may lack the requisite sensitivity when donor cell survival is low.

PCR is now a routine method for the detection of very low levels of specific nucleotide sequences; this has revolutionized both experimental research and clinical diagnoses. The sensitivity and specificity of PCR depends on the optimal design of specific primers. However, false negatives are still possible as the template concentration is reduced. Wang et al. (2002) evaluated engraftment efficiency in human–mouse chimeras using real-time PCR and successfully measured the engraftment of male human liver cells in female mouse liver tissues when the DNA level (approximate cell fraction) was 0.125%–0.257%. Nested PCR has the potential to provide reliable results in cases of low template number using a two-step reaction. In the first step, conditions can be optimized for maximal replication efficiency using species-specific PCR primers to enhance sensitivity. In the second step, nested or inner primers are used to specifically amplify only target products, thus improving specificity. After two-step amplification, many templates that were undetectable with one-step PCR may reach the level of detection (AbouLaila et al., 2010). Byproducts may also be undetectable due to the nonspecific amplification (Figure 6). Therefore, nPCR is a simple, convenient, and reliable method for the rapid screening of interspecific chimeras compared to real-time PCR.

In this study, the nPCR primers were specific to the human SRY gene, as indicated by comparing sequenced products to GenBank. No products or byproducts were found using female human or female mouse DNA as the template (i.e. no false positives); this confirmed the high specificity of the nPCR primers. Furthermore, the nPCR was sensitive enough to detect human SRY

![Figure 6. The procedure for one-step PCR and nPCR.](image-url)
DNA in a 1:10,000 male human–female mouse mixture and thus could be used to detect interspecific chimeras and optimize animal models of cell transplantation. In the practical test, one-step PCR detected only one chimera in 88 samples, while nPCR detected 14 chimeras that were subsequently confirmed by FISH and immunohistochemistry. The positives of nPCR were higher than one-step PCR. Comparing the conditions of nPCR and one-step PCR, we learned that nPCR has higher specificity and sensitivity (Table). Our nPCR assay was superior to one-step PCR in detecting human SRY-positive cells in interspecific chimeras, confirming the improved specificity and sensitivity of this nPCR method (Lin et al., 2010). Indeed, our nPCR method was as reliable as FISH or immunohistochemistry, but is much more practical for high-throughput screening.

In our research, we found that nPCR is a simple and quick method for human cell detection in human–mice chimeras, whereas FISH and immunohistochemistry are time-consuming and labor-intensive.

In conclusion, the designed nPCR primers were specific to the human SRY gene. This meant that our primers did not react to the DNA of female humans or mice. Furthermore, the sensitivity of the proportion of human to mouse was more than 1:10,000, which was 100 times higher than conventional one-step PCR (human:mice = 1:100). Therefore, nPCR established that we can detect human cells in human–mice chimeras.

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**Table.** Comparison of one-step PCR and nPCR.

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<th>One-step PCR</th>
<th>nPCR</th>
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<tr>
<td>Template</td>
<td>2 µL DNA template (sample)</td>
<td>0.5 µL amplified PCR products</td>
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<tr>
<td>Cycles</td>
<td>35</td>
<td>27</td>
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<td>Annealing temperature</td>
<td>65 °C</td>
<td>65 °C</td>
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<tr>
<td>Primer concentrations</td>
<td>10 pmol/µL</td>
<td>10 pmol/µL</td>
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<tr>
<td>Result</td>
<td>1/88 and some byproducts due to the nonspecific amplification shown in Figure 2</td>
<td>14/88 and few byproducts due to the nonspecific amplification shown in Figure 3</td>
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<tr>
<td>Possibility of low or high positives</td>
<td>1. The concentrations of target sequences of DNA from a large complex mixture of DNA may be very low. 2. The number of cycles may be low. 3. The 65 °C annealing temperature may be high. The positives may be increased as the sample concentrations are increased, the cycles are raised, and the annealing temperature is reduced. However, nonspecific amplification products would increase at the same time. Therefore, the target products’ 270-bp band, amplified with one-step PCR, could not reach the level of detection.</td>
<td>The concentrations of target products from one-step PCR products are high. The nested primers are used to specifically bind within the target products. The primers of nPCR bind within the one-step PCR products. If the nonspecific products were amplified by one-step PCR, the probability is very low in nPCR. After two-step amplification, target products reach the level of detection.</td>
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**References**


