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TITLE:

Quantification of Circulating Pig-Specific DNA in the Blood of a Xenotransplantation Model

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KEYWORDS:

specific primers, circulating pig-specific DNA, qPCR, xenotransplantation, biomarker,immune rejection

SUMMARY:

In this protocol, porcine specific primers were designed, plasmids-containing porcine specific DNA fragments were constructed, and standard curves for quantitation were established. Using

species-specific primers, cpsDNA was quantified by qPCR in pig-to-mouse cell transplantation models and pig-to-monkey artery patch transplantation models.

ABSTRACT:

Xenotransplantation is a feasible method to treat organ failure. However, how to effectively monitor the immune rejection of xenotransplantation is a problem for physicians and researchers. This manuscript describes a simple and effective method to monitor immune rejection in pig-to-mouse cell transplantation models and pig-to-monkey artery patch transplantation models. Circulating DNA is a potentially non-invasive biomarker for organ damage. In this study, circulating pig-specific DNA (cpsDNA) was monitored during xenograft rejection by quantitative real-time PCR (qPCR). In this protocol, porcine specific primers were designed, plasmids-containing porcine specific DNA fragments were constructed, and standard curves for quantitation were established. Species-specific primers were then used to quantify cpsDNA by qPCR in pig-to-mouse cell transplantation models and pig-to-monkey artery patch transplantation models. The value of this method suggests that it can be used as a simple, convenient, low cost, and less invasive method to monitor the immune rejection of xenotransplantation.

INTRODUCTION:

Organ failure is one of the main causes of death[1]. Transplantation of cells, tissues and organs is an effective way to treat organ failure[2]. Nevertheless, the shortage of donor organs limits the clinical application of this method[3,4]. Studies have shown that pigs can be used as a potential source of human organs for clinical transplantation[5,6]. However, cross-species organ transplantation faces dangerous immune rejection. Therefore, it is crucial to monitor the immune rejection of xenotransplantation. Currently, clinical monitoring of immune rejection depends mainly on the patient's signs and symptoms, as well as laboratory tests (e.g., biopsy, immunobiochemical analysis, and ultrasound)[7-9]. However, these monitoring methods have many disadvantages. The signs and symptoms of immune rejection in patients usually appear late[10], which is not conducive to early detection and early intervention; biopsy has the disadvantage of being invasive[11], which is not easy for patients to accept; immunobiochemical analysis lacks sensitivity or specificity, and ultrasound is auxiliary and expensive. Therefore, it is urgent to find an effective and convenient method to monitor the immune rejection.

Circulating DNA is an extracellular type of DNA found in blood. Mandel and Metais[12] first reported the presence of circulating DNA in peripheral blood in 1947. Under normal physiological conditions, circulating DNA in the blood of healthy people is relatively low at baseline. However, in some pathologies, such as tumors, myocardial infarction, autoimmune diseases, and transplant rejection, the level of circulating DNA in the blood can be significantly increased[13,14] due to the massive release of circulating DNA caused by apoptosis and necrosis. The origin of circulating DNA is associated with apoptosis and necrosis[15], which are characteristic of xenograft rejection[16].

Circulating DNA has been proven to be a minimally-invasive biomarker for detecting cancers[17-19]. High through-put sequencing of donor-derived circulating DNA is reliable for the detection of

rejection after organ transplantation[20,21]. However, this method requires a high concentration and quality of extracted DNA. The DNA requirements in addition to the high cost and time-consumption make this method ineligible for routine clinical use. Donor-derived circulating DNA can be precisely quantified by quantitative real-time PCR (qPCR), which is both specific and sensitive. Therefore, quantifying porcine circulating DNA by qPCR is a feasible method to monitor the immune rejection of xenotransplantation. This is less invasive, highly sensitive and specific, low cost, and time-saving. Pigs and human beings are genetically separate with quite different genomic sequences (**Figure 1**). Therefore, circulating porcine DNA can be released into the recipient's blood post-xenotransplantation because of xeno-rejection. CpsDNA could be precisely quantified by qPCR with species-specific primers in the recipient's blood. Previously, we have demonstrated the rationale and feasibility of cpsDNA as a biomarker for xenotransplantation[22,23]. Here, we disclose more experimental tips and details. The experiment consists of the following steps. Firstly, porcine specific primers were designed, and genomic DNA was isolated, which were used to verify the specificity of the primers by regular PCR. Secondly, constructing the standard curve of cpsDNA and isolating cpsDNA from the sample blood. Finally, the circulating pig-specific DNA was quantified using real-time PCR.

PROTOCOL:

All experiments were performed in accordance with the relevant guidelines and regulations of the Institutional Review Board of Shenzhen Second People's Hospital, First Affiliated Hospital of Shenzhen University.

1. Design porcine specific primers

1.1. Perform whole-genome BLAST analysis to identify porcine specific genes that were different from those of humans, monkeys or mouse, using NCBI software (www.ncbi.nlm.nih.gov).

1.2. Design primers according to 19 pig-specific genes (**Table 1**) using software. Purchase them commercially (**Table of Materials**).

2. Isolating genomic DNA

NOTE: The genomic DNA (including blood from pigs, monkeys, volunteer humans, monkeys with pig grafts, and mice with porcine cells) were extracted using a commercial genomic DNA extraction kit (**Table of Materials**).

2.1. Place 500 µL of the whole blood of above samples into different microcentrifuge tubes, respectively.

2.2. Add 20 µL of protease K and 500 µL of lysate into the above microcentrifuge tubes, and then thoroughly shake and mix.

2.3. Put these microcentrifuge tubes in a water bath at 56 °C for 10 min and shake 2-3 times during this process until the solution becomes clear.

2.4. Centrifuge briefly to remove the liquid beads from the inner wall of the tube covers. Add 500 µL of anhydrous ethyl alcohol and shake thoroughly.

2.5. Transfer the mixture into the adsorption column, centrifuge at 6,200 x *g* for 2 min, and finally, discard the waste liquid.

2.6. Add 800 µL of rinse solution to each adsorption column; centrifuge for 1 min at 6,200 x *g*. Place the columns at room temperature for a few minutes to dry the remaining rinse solution.

NOTE: The rinse solution is provided by the manufacturer.

2.7. Transfer the adsorption columns to another clean centrifugal tube, add 50 µL of elution buffer to the middle of the adsorption films, and place them at room temperature for 2-5 min. Centrifuge 6,200 x *g* for 1 min.

NOTE: The elution buffer is provided by the manufacturer, but the TE buffer, containing 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA, can also elute the DNA.

2.8. Store the DNA solution at -20 °C.

3. Verify the specificity of the primers

NOTE: Species specificities of the above 19 primers were confirmed by PCR, which was performed using polymerase (**Table of Materials**) and primers presented in **Table 1**.

3.1. Prepare the pre-mixed solution of 12.5 µL of 2x polymerase (**Table of Materials**), 1 µL of 5' primer (10 µM), 1 µL of 3' primer (10 µM), and 8.5 µL of ddH_2O. Prepare the mix including 2 extra samples.

3.2. Split 23 µL of pre-mixed solution into 0.6 mL microcentrifuge tubes, add 2 µL of genomic DNA, and carefully cap the tubes. Then mix and centrifuge slightly.

3.3. Place the 0.6 mL microcentrifuge tubes into the PCR-cycler and perform the following: Denaturation: 95 °C for 5 s; Annealing: 60 °C for 30 s; extension:72 °C for 30 s.

3.4. Perform agarose electrophoresis.

3.4.1. Weigh 1.2 g of agarose into a flask containing 100 mL of 1x TAE and boil it for 5 min in the microwave. Add 5 µL of nucleic acid dye (**Table of Materials**) into the flask after it cools to about 70 °C. Pour it into the plate along the edge slowly, and place it at room temperature until

it solidifies into a gel.

3.4.2. Add 5 µL of sample and 2-Log DNA Ladder (0.1–10.0 kb) or DNA marker I (0.1–0.6 kb), which contain 1 µL of 6x DNA loading buffer, into the agarose gel. Then electrophorese at 120 mA until the bands are separated.

3.4.3. Visualize the agar gel containing DNA fragments with an ultraviolet imager.

NOTE: Using the PCR, the primers specific for amplified porcine genomic DNA were identified. Further, two species-specific primers were determined to amplify pig DNA in the pig-to-monkey artery patch and the pig-to-mouse cell transplantation models.

4. Standard curve of cpsDNA

4.1. Transform the pMD19-T plasmid containing a fragment of porcine DNA into DH5a, which could be specifically amplified by primer #4 or primer #11 (**Table 1**). Screen for positive bacteria using 50 µg/mL ampicillin.
Primer #4 in human/monkey cohort (forward: 5'-TTCAATCCCA CTTCTTCCACCTAA-3', reverse: 5'-CTTCATTCCATCTTCATAATAAC CCTGT-3')
Primer #11 for mouse model (forward: 5'-TGCCGTGGTTTCC GTTGCTTG-3', reverse: 5'-TCACATTTGATGGTCGTCTTGTCGTC T-3')

NOTE: Details of all the primers can be found in **Table 1**.

4.2. Harvest the above plasmids following the protocol below.

4.2.1. Isolate a single colony from a freshly streaked selective plate and inoculate a culture of 1- 5 mL of LB medium containing the appropriate selective antibiotic. Incubate for ~12-16 hours at 37 °C with vigorous shaking (~ 300 rpm).

4.2.2. Centrifuge at 10,000 x *g* for 1 minute at room temperature. Decant or aspirate and discard the culture media.

4.2.3. Add 250 µL of Solution I/RNase A. Vortex or pipet up and down to mix thoroughly. Complete resuspension of cell pellet is vital for obtaining good yields.

NOTE: RNase A must be added to Solution I before use.

4.2.4. Transfer suspension into a new 1.5 mL microcentrifuge tube. Add 250 µL of Solution II. Invert and gently rotate the tube several times to obtain a clear lysate. A 2-3 minute incubation may be necessary.

NOTE: Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity. Do not allow the lysis reaction to proceed more than 5 minutes.

4.2.5. Add 350 µL of Solution III. Immediately invert several times until a flocculent white precipitate forms.

NOTE: It is vital that the solution is mixed thoroughly and immediately after the addition of Solution III to avoid localized precipitation.

4.2.6. Centrifuge at maximum speed (≥13,000 x *g*) for 10 minutes. A compact white pellet will form. Promptly proceed to the next step.

4.2.7. Insert a DNA mini column into a 2 mL collection tube.

4.2.8. Transfer the cleared supernatant from 4.2.7 by carefully aspirating it into the DNA mini column. Be careful not to disturb the pellet and that no cellular debris is transferred to the DNA mini column.

4.2.9. Centrifuge at maximum speed for 1 minute. Discard the filtrate and reuse the collection tube.

4.2.10. Add 500 µL of HBC Buffer. Centrifuge at maximum speed for 1 minute. Discard the filtrate and reuse collection tube.

NOTE: HBC Buffer must be diluted with 100% isopropanol before use.

4.2.11. Add 700 µL of DNA Wash Buffer. Centrifuge at maximum speed for 1 minute. Discard the filtrate and reuse the collection tube.

NOTE: DNA Wash Buffer must be diluted with 100% ethanol prior to use.

4.2.11.1. Repeat for a second DNA wash buffer wash step.

4.2.12. Centrifuge the empty DNA mini column for 2 minutes at maximum speed to dry the column matrix.

NOTE: It is important to dry the DNA mini column matrix before elution. Residual ethanol may interfere with downstream applications.

4.2.13. Transfer the DNA mini column to a clean 1.5 mL microcentrifuge tube.

4.2.14. Add 30-100 µL of Elution Buffer or sterile deionized water directly to the center of the column membrane.

NOTE: The efficiency of eluting DNA from the DNA Mini column is dependent on pH. If using sterile deionized water, make sure that the pH is around 8.5.

4.2.15. Let sit at room temperature for 1 minute.

4.2.16. Centrifuge at maximum speed for 1 minute.

NOTE: This represents approximately 70% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration.

4.3. Verify the above plasmids using by double restriction enzyme digestion using EcoR I (15 U/µL) and Bam H1(15 U/µL)[22].

4.3.1. Perform the following on ice. Prepare the reaction system of 1 µL of EcoR I (15 U), 1 µL of Bam H1 (15 U), 2 µL of 10x Buffer, 1 µg of plasmid; add ddH_2O to 20 µL of total volume. Incubate in a water bath at 37 °C for 2 hours.

4.3.2. Separate the digested products by 1% agarose followed by electrophoresis and expose to UV light as before.

4.4. Dilute the concentrated plasmid into 2 x 10^{11} copies/mL for the starting standard solution using ddH_2O. Take the plasmid (P2) containing fragment of porcine DNA and calculate the following:

4.4.1. Calculate the number of plasmids in 1 mL of starting standard solution: N = (2 x 10^{11})/ (6.02 x 10^{23}) mol.

4.4.2. Calculate the molecular weight of P2: M = 2810 bp x 650 D/bp = 2810 x 650 D (g/mol).

4.4.3. Calculate the mass of P2: m=N*M= (2 x 10^{11})/ (6.02 x 10^{23}) mol x 2810 x 650 g/mol.

4.4.4. Calculate the volume of P2: V = m/C = [(2 x 10^{11})/(6.02 x 10^{23}) x 2810 x 650] g/C, where C is the concentration of P2 (ng/µL), which is measured by a spectrophotometer.

4.5. Dilute the starting standard solution serially 10-fold into 2 x 10^{10} copies/mL, 2 x 10^{9} copies/mL, 2 x 10^{8} copies/mL, 2 x 10^{7} copies/mL…, and 2 x 10^{0} copies/mL for standard solution using ddH_2O. Vortex thoroughly during dilution.

4.6. Establish the standard curve.

4.6.1. Prepare reaction system of qPCR (all steps are protected from light): Add the pre-mixed solution which contains 325 µL of qPCR Mix (**Table of Materials**), 13 µL of 5' primer, 13 µL of 3' primer, and 169 µL of ddH_2O into a 1.5 mL microcentrifuge tube, which was then thoroughly mixed and slightly centrifuged.

4.6.2. Split the 40 µL above pre-mixed solution into an 8-tube strip and add 10 µL of standard

DNA of different concentrations. Cap carefully, mix and centrifuge slightly.

4.6.3. Put the 8-tube strip into the qPCR machine following the procedure shown in **Figure 2**.

5. Isolate circulating DNA from the blood samples

5.1. Using EDTA tubes, collect blood samples at different time points in the pig-to-monkey artery patch models and the pig-to-mouse cell transplantation models.

5.2. Collect blood samples of about 400 µL (from the pig-to-monkey artery patch models) or 100 µL (from the pig-to-mouse cell transplantation models) and transfer to 1.5 mL centrifuge tubes. Remove the blood cells from the blood samples by centrifugation at low temperature and high speed (3,000 x *g*, 4 °C, 5 min).

5.3. Transfer the supernatant to a new 1.5 mL centrifuge tube and remove the cell debris by centrifugation at 16,000 x *g* for 10 min.

5.4. Transfer the supernatant to a new 1.5 mL centrifuge tube. Extract the circulating DNA from the above supernatant using a commercial serum/circulating DNA extraction kit following protocol 2 of the isolating genomic DNA manufacturers' protocol.

5.5. Condense the volume of circulating DNA to 40 µL and store at -20 °C.

6. Quantitation of circulating pig-specific DNA

6.1. Prepare the pre-mixed solution of 25 µL of qPCR Mix (**Table of Materials**), 1 µL of 5' primer, 1 µL of 3' primer, 10 µL of Sample DNA, and 13 µL of ddH_2O. Prepare the mix including 2 extra samples.

6.2. Split the 40 µL pre-mixed solution into an 8-tube strip, and add 10 µL of sample DNA, followed by careful capping. Prepare two more reactions than needed.

6.3. Put the 8-tube strip into the qPCR machine following the same procedure as the standard curve. It is better to run a standard first to make sure the reaction is critical for quantification in advance. The procedure of the reaction system of qPCR was shown in **Figure 2**.

REPRESENTATIVE RESULTS:

In this protocol, porcine specific primers were designed, plasmids-containing porcine specific DNA fragments were constructed, and standard curves for quantitation were established (**Figure 3**). Species specificities of the 19 primers were confirmed by PCR. Species-specific primers (primer #4 and primer #11) were then used to quantify cpsDNA by qPCR in pig-to-mouse cell transplantation models and pig-to-monkey artery patch transplantation models.

Agarose electrophoresis was used to isolate amplified DNA fragments from the above samples,

which is then visualized in an ultraviolet imager. Using PCR, the primers specific for amplified porcine genomic DNA was first identified (**Figure 4A**). Further, certain species-specificities of these primers that specifically amplified pig DNA in the cohort of monkey/human genomic or in the cohort of mouse genomic DNA were confirmed (**Figure 4B**). Finally, two species-specificities primers were further proven to specifically amplify pig DNA in the pig-to-monkey artery patch and/or pig-to-mouse cell transplantation models, respectively (**Figure 4C**).

Figure 1: Gene identity between pig (*sus scrofa*, ssc) and human (*homo sapiens*, hsa) or monkey (*Macaca fascicularis*, mfa). The gene sequences from three different species were compared by BLAST analysis. BLAST sequence analysis used genomic annotation information (the mRNA sequence) from the three species downloaded from NCBI (www.ncbi.nlm.nih.gov). The mRNA sequences of human, monkey, and pig genes were 139116, 65927, and 71498, respectively[22].

Figure 2: The procedure for the qPCR reaction.

Figure 3: Establishment of the standard curve for absolute quantification. The amplification plots, the melt curve plots, and the standard curve views of (**A**) primer #4 and (**B**) primer #11 from the qPCR machine (see **Table of Materials**) are exhibited. A good standard (R value close to 1, amplification efficiency is within 100%±5%) could be used for up to half a year.

Figure 4: Regular PCR validates specificity of porcine specific primers. (**A**) The pig genomic DNA fragments were amplified by primers 1-19. (**B**) The human/monkey/mice genomic DNA fragment could not be amplified by some primers (primer #4 and #11). The stars (*) indicate no amplifications in human/monkey genomic DNA. The pound sign (#) indicates no amplification in mouse genomic DNA. (**C**) The two species-specificities primers (primer #4 and #11) were further proven to specifically amplify pig DNA in the pig-to-monkey artery patch and/or pig-to-mouse cell transplantation models, respectively.

DISCUSSION:

Quantifying porcine circulating DNA represents a feasible approach to monitor the immune rejection of xenotransplantation. Gadi et al.[24] found that donor-derived circulating DNA (ddcfDNA) content in the blood of patients with acute rejection was significantly higher than that of patients without rejection. These studies suggest that ddcfDNA may be a common biomarker for monitoring organ graft damage. In recent years, qPCR has increasingly been applied to the analysis of nucleic acids because of its simple operation, high degree of automation, high sensitivity, good specificity, and low cost.

In this study, porcine specific primers were designed based on the results of bioinformatics analysis. Their specificity was verified by regular PCR. The cpsDNA of the blood samples of the pig-to-monkey artery patch models and the pig-to-mouse cell transplantation models could be precisely quantified by real-time quantitative PCR (qPCR)with species-specific primers. The main advantages of the approach are as follows. Firstly, based on the highly specific primers, this method of quantifying DNA is highly sensitive and specific. In addition, the protocol of the

approach is very simple to perform, which consisted of isolation of DNA, design of specific primers, and real-time PCR analysis over 1 working day, which is timesaving. It is a non-invasive methods that starts from a small volume of serum or plasma material. Meanwhile, we have demonstrated that this method is highly reproducible[22]. In contrast to high-throughput sequencing and flight mass spectrometry, this method is low cost.

Monitoring immune rejection by quantification of circulating pig-specific DNA in the blood of pig-to-monkey artery patch models and pig-to-mouse cell transplantation models using qPCR has laid the foundation for the basic research and the clinical application of porcine-human xenotransplantation.

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DISCLOSURES:

The authors report no conflicts of interest.

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Figure 1

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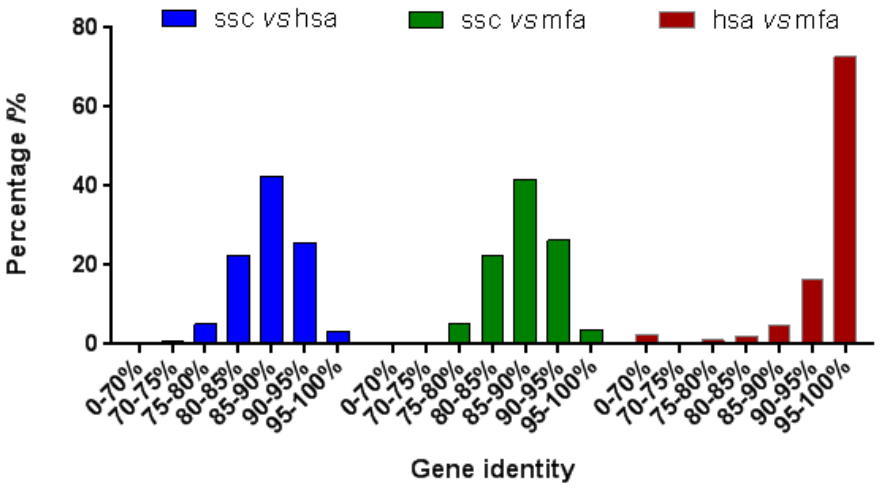

Figure 2

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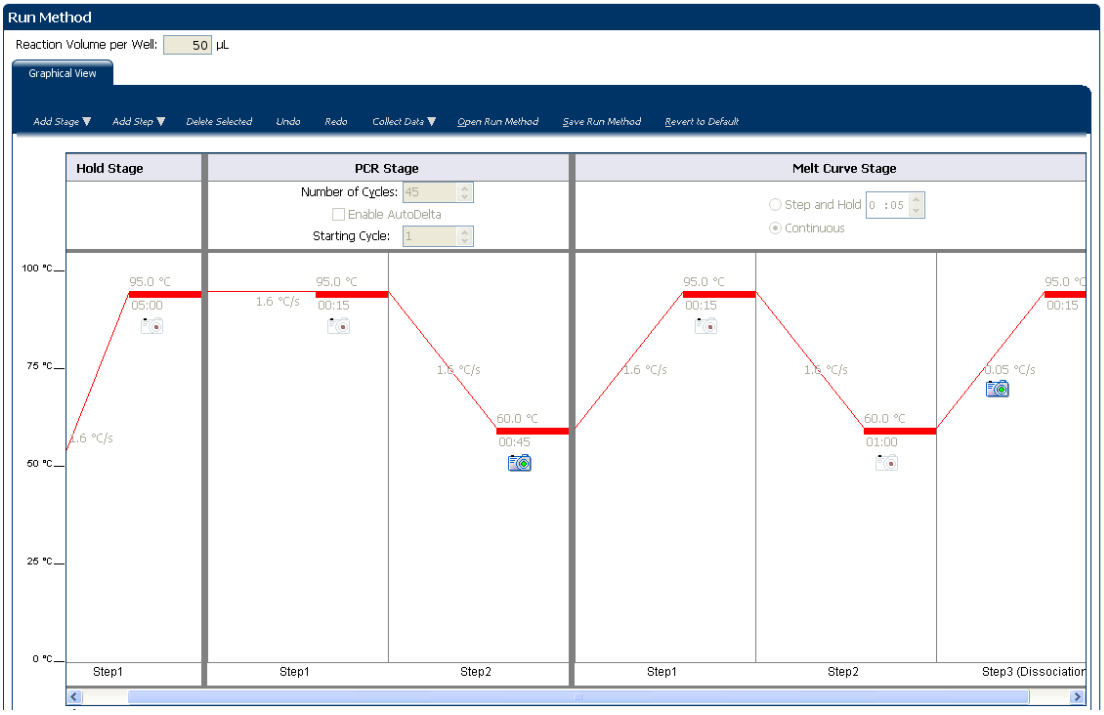

Figure 3

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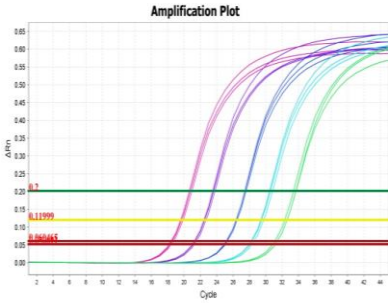

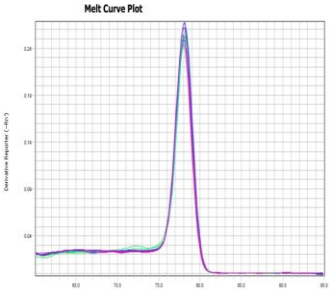

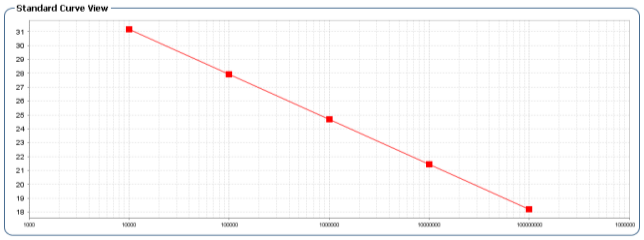

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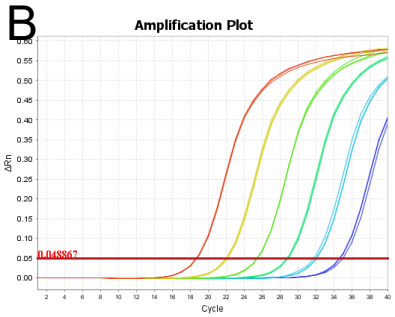
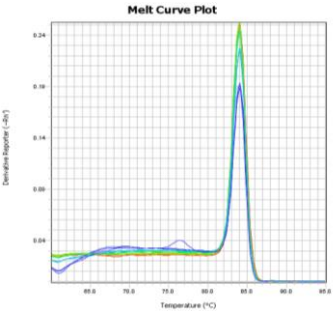
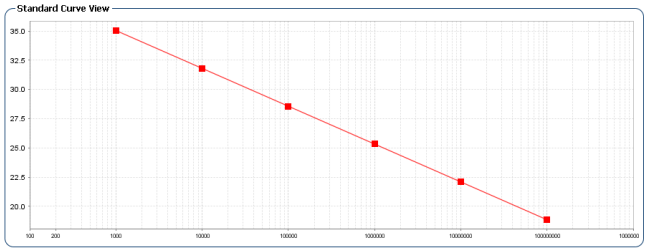
B



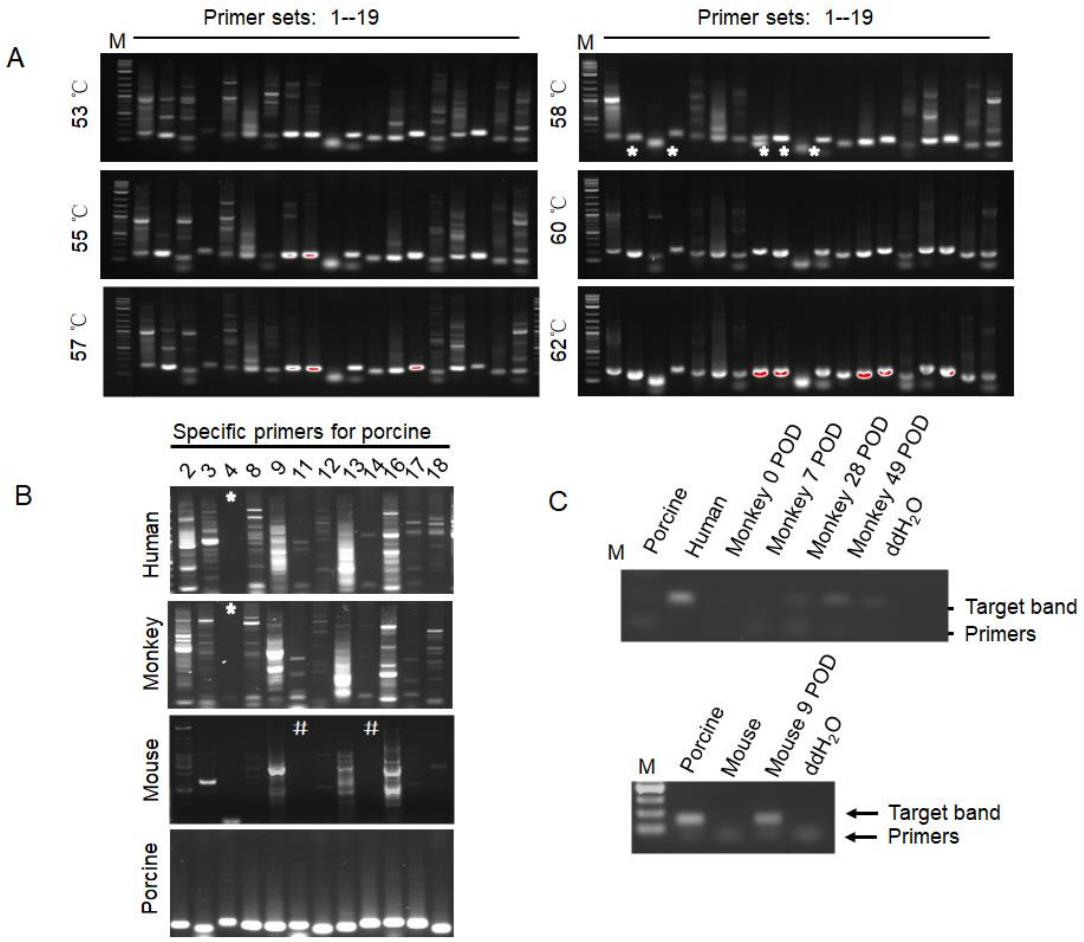




Reporter	Target	Slope	Y-Intercept	R(superscript 2)	Efficiency(%)
SYBR	Target 1	-3.239	44.767	0.999	103.563

Figure 4

Click here to access/download;Figure;Figure4.pdf



Click here to access/download;Table;Table 1.xlsx

Table 1:Details of primers used in this study.

NO.	Genebank access number	duroc[a]_location	description	Forward primer（5'—3'）	Reverse primer（5'—3'）	product size (bp)
1	XM_005662127.1	chromosome_03:32653946-32654670	PREDICTED: Sus scrofa protamine 2 (PRM2), transcript variant X1	CAAGGATTATGAGGCAGGAATGGATTGTG	AGCGTGGAGGGAACCAAGGGCAAGG	145
2	NM_001145219.1	chromosome_03:32653946-32654670	Sus scrofa galectin-related inter-fiber protein	CCACTTGTCCCGAGGCTCCC	GCTGCGAAGGCATTGCGTTTAG	120
3	XM_005655747.1	chromosome_06:1597985-1607074	PREDICTED: Sus scrofa uncharacterized LOC102163997	TGTCTGAACACGGACGGACAGGCTACT	CCCAAGGTGACCCTGACTCTGGAGC	86
04[b]	**XM_005661120.1**	**chromosome_02:48903418-48904922**	**PREDICTED: Sus scrofa basic salivary proline-rich protein 3-like**	**TTCAATCCCACTTCTTCCACCTAA**	**CTTCATTCCATCTTCATAATAACCCTGT**	**118**
5	XM_005668038.1	chromosome_10:35225831-35229726	PREDICTED: Sus scrofa translation initiation factor IF-2-like	CCGCGAGGAACTCCCATACA	GGATTAGGAAAGAAAGGCAGCAAG	116
6	XM_003484024.2	chromosome_18:20260826-20270718	PREDICTED: Sus scrofa small lysine-rich protein 1 (SMKR1)	ACTATGCCGCAGCAGTGACAACA	CCGTGGGAGTGGCTCAAGAAAA	105
7	XM_005660594.1	chromosome_02:48903418-48904922	PREDICTED: Sus scrofa uncharacterized LOC100737725	CAGCCCTCACTGTGGACATG	AAAGCCTCGTGCGAAATAGC	96
8	XM_005653889.1	chromosome_12:5696213-5698343	PREDICTED: Sus scrofa small integral membrane protein 6 (SMIM6)	TGGCAGGAGGACCTACAGCACAACCC	GCAGGGAGTAAACCAAACGTAATAGCGAACA	119
9	XM_005668771.1	chromosome_12:5696213-5698343	PREDICTED: Sus scrofa formin-like protein 5	GGGGTTTAGTTCCAATGGTCTGC	AGTGGCTTGAGTGGCTCCTGTCT	119
10	XM_005659754.1	chromosome_01:145796994-145798743	PREDICTED: Sus scrofa uncharacterized LOC102161119	ATGTATGTGGGAATGTTCTCCACAA	TTGTGGAGAACATTCCCACATACAT	102
11[c]	**XM_005654637.1**	**chromosome_02:48903418-48904922**	**PREDICTED: Sus scrofa src substrate cortactin-like**	**TGCCGTGGTTTCCGTTGCTTG**	**TCACATTTGATGGTCGTCTTGTCGTCT**	**119**

12	XM_005661911.1	chromosome_03:32653946-32654670	PREDICTED: Sus scrofa cohesin subunit SA-3-like	GCTACTTACAAACCTCCCTCCTCCTT	TCAGTCTCGCTGATGCCTTATCC	83
13	XM_005663733.1	chromosome_04:142518394-142549996	PREDICTED: Sus scrofa collagen alpha-1(XXIV) chain	GGGAGGTGATGGTGGCAGGTA	GGAGGGACAGAGGTGTTGAGGAA	101
14	NM_001003925.1	chromosome_05:23305080-23306163	Sus scrofa ovarian and testicular apolipoprotein N	TCTGGGTCTCCACCGCTTCTTT	CATTTGTACTGACTACTCCAATCCCTTGT	118
15	XM_005658989.1	gi|333778929|ref|NW_003541098.1|:7-1376	PREDICTED: Sus scrofa leucine rich repeat containing 45	AGCCAGGTGAAGGCGGCGGCACT	CCAGACGCACTTGACTCTTGGCCTTGAGA	82
16	XM_005657990.1	chromosome_12:5696213-5698343	PREDICTED: Sus scrofa integrin alpha-E-like	AATAATGCTGCCTGCCCTGCTC	CCCACAACCCAAACTGCTTCCTA	144
17	XM_005659796.1	chromosome_01:145796994-145798743	PREDICTED: Sus scrofa uncharacterized LOC102163334	TCTGCTGAGCCACAAGGGAATGT	AAACGGACCTGAAGAAGCCGAAA	132
18	NM_213942.1	chromosome_09:74195820-74207816	Sus scrofa complement component 4 binding protein, alpha	AAATAGATGAGCAGCCAACCAAGA	CCATTAGACCGCCAGGGAAC	82
19	XM_005660599.1	chromosome_02:48903418-48904922	PREDICTED: Sus scrofa glutamine-dependent NAD(+) synthetase-like	TGCCGTGGTTTCCGTTGCTTG	TCACATTTGATGGTCGTCTTGTCGTCT	119

a Durocbreed pigs.

b/c Primers used in the monkey and/or mouse models.

Click here to access/download;Table of Materials;Table_of_Materials July 21 2020 DYY.xlsx

Name of Material/Equipment	Company	Catalog Number
Agarose	Sigma，USA	V9005100
Bam H1	Takara Bio, China	1010S
centrifuge tube	Axygen, China	MCT-150-C
centrifuge tube	Axygen, China	MCT-060-C
C57BL/6 Mice	Medical Animal Center of Guangdong Province, China	
centrifuge	Thermo Scientific	ST16R
DNA Ladder	NEB，China	N3200S
DNA marker(TIANGEN)	Tiangen, Beijing, China	MD101-02
DNA Mini Column(HiBind DNA Mini Columns)	Omega Biotech, USA	DNACOL-01
DNA loading buffer	Solarbio, China	D1010
E.Z.N.A.Plasmid DNA Mini Kit I and E.Z.N.A. Plasmid DNA Mini Kit II	Omega Biotech, USA	D6942 D6943 D6943
EcoR I	Takara Bio, China	1040S
EB	Thermo Fisher Scientific, USA	15585011
Female Bama mini pigs	BGI Ark Biotechnology	
Genomic DNA Extraction Kit Ⅰ	Tiangen, Beijing, China	DP304-02
Green Master Mix(ThunderBird SYBR)	Toybo, Osaka, Japan	QPS201
Gel Doc XR	Bio-Rad, Hercules, USA	
Male cynomolgus monkeys	Guangdong Landau Biotechnology, China	
Nucleic acid dye(Gelred)	Biotium,Fremont, USA	42003
polymerase(ExTaq)	Takara, Dalian, China	RR001A
pMD19-T plasmid	Takara, Dalian, China	D102A
qPCR machine	Applied Biosystems ViiA7, Waltham, USA	
Serum/Circulating DNA Extraction Kit	Tiangen, Beijing, China	DP339
TAE	Solarbio, China	T1060

Comments/Description

8~10 weeks

0.1–10.0 kb
0.1–0.6 kb

2~4 months

8 years

Rebuttal Letter
Click here to access/download;Rebuttal Letter;answer of the comments July-23 2020 MLS.docx

To the Editor,

Our coauthors and I thank you and the reviewers for your interest in publishing this study. We have carefully considered all of the comments made by the reviewers and have addressed them below. We have made the necessary modifications to the manuscript, and tracked the changes.

We hope the manuscript is now fully acceptable to you for publication in your journal. We look forward to hearing from you when you have had an opportunity to consider our responses and the revisions we have made.

Yours faithfully,

Lisha Mou

Editorial comments:

Q: 1. Protocol Language: Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

(1) Examples NOT in the imperative: 1.1, 1.2, 2.8, 4.3, etc.

(2) Lines 103-105 should be a note.

A: Thanks for your suggestion, we revised the manuscript as your suggestions.

(1) Previous：1.1 Whole-genome BLAST analysis was used to identify 19 porcine specific genes that were different from those of humans or monkeys, using Software of NCBI (www.ncbi.nlm.nih.gov).

Revised：1.1 Perform whole-genome BLAST analysis to identify porcine specific genes that were different from those of humans, monkeys or mouse, using Software of NCBI (www.ncbi.nlm.nih.gov). Page 4, Line 99-101.

(2) Previous: 1.2 Primers were designed according to 19 pig-specific genes (Table 1) using software of Primer 5 and were produced by The Beijing Genomics Institute.

Revised: 1.2 Design the primers according to 19 pig-specific genes (Table 1) using software of Primer 5. Page 4, Line 102-104.

(3) Previous: 2.1 500 μL whole blood of the above samples was first placed into different EP tubes, respectively.

Revised: 2.1 Place 500 μL the whole blood of above samples into different EP tubes, respectively. Page 4, Line 111-112.

(4) Previous: 2.5 The mixture was then transferred into the adsorption column, centrifuged at 8000rp for 2 min, and finally, the waste liquid was discarded.

Revised: 2.5 Transfer the mixture into the adsorption column, centrifuged at

6200×g for 2 min, and finally, the waste liquid was discarded. Page 5, Line 119-120.

(5) Previous: 2.8 The DNA solution was collected into the centrifuge tubes and stored at -20℃.

Revised: 2.8 Store the DNA solution at -20℃. Page5, Line130.

(6) Previous: 5.4 The supernatant was transferred to a new 1.5Ml centrifuge tubes, respectively. Circulating DNA was extracted from the above supernatant using a Serum/Circulating DNA Extraction Kit as the protocol 2 of isolating genomic DNA, per the manufacturers' protocol.

Revised: 5.4 Transfer the supernatant to a new 1.5mL centrifuge tubes, respectively. Extract the circulating DNA from the above supernatant using a Serum/Circulating DNA Extraction Kit as the protocol 2 of isolating genomic DNA, per the manufacturers' protocol. Page 10, Line 263-266.

(7) Previous: 5.5 The volume of circulating DNA was eventually condensed to 40μL and stored at -20℃.

Revised: 5.5 Condense the volume of circulating DNA to 40μL and stored at -20℃. Page 10, Line 267-268.

Q: 2. Protocol Detail: Please note that your protocol will be used to

generate the script for the video, and must contain everything that you would like show in the video. Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc.) have been added to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:

1) 2.5, 2.6 : Mention centrifuge speeds in x g.

A: (1) Previous: 2.5 The mixture was then transferred into the adsorption column, centrifuged at 8000rp for 2 min, and finally, the waste liquid was discarded.

Revised: 2.5 Transfer the mixture into the adsorption column, centrifuged at 6200×g for 2 min, and finally, the waste liquid was discarded. Page 5, Line 119-120.

(2) Previous: 2.6 Add 800μL rinse solution to each adsorption column; centrifuge for 1min at 8000rpm. Place the columns at room temperature for a few minutes to dry the remaining rinse solution.

Revised: 2.6 Add 800μL rinse solution to each adsorption column; centrifuge for 1min at 6200×g. Place the columns at room temperature for a few minutes to dry the remaining rinse solution. Page5, Line 121-123.

2) 2.6: what is the composition of rinse solution, elution buffer etc.?

A: **(1)** Previous: 2.6 Add 800μL rinse solution to each adsorption column; centrifuge for 1min at 8000rpm. Place the columns at room temperature for a few minutes to dry the remaining rinse solution.

Revised: 2.6 Add 800μL rinse solution to each adsorption column; centrifuge for 1min at 6200×g. Place the columns at room temperature for a few minutes to dry the remaining rinse solution.

Note: the rinse solution is provided by the manufacturer. Page5, Line 121-124.

(2) Previous: 2.7 Transfer the adsorption columns to another clean centrifugal tube, add 50μL elution buffer to the middle of the adsorption films, and place them at room temperature for 2-5 min. And then centrifuge 8000rp for 1 min.

Revised: 2.7 Transfer the adsorption columns to another clean centrifugal tube, add 50μL elution buffer to the middle of the adsorption films, and place them at room temperature for 2-5 min. And then centrifuge 6200×g for 1 min.

Note: the elution buffer is provided by the manufacturer, but the TE buffer, containing 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA, can also elute the DNA. Page 5, Line 125-129.

3) 4.2: Citing a product manual is not sufficient for filming.

A: Previous: 4.2 Harvest the above plasmids following a protocol, such as Omega Biotech.

Revised: 4.2 Harvest the above plasmids following the protocol below.

4.2.1. Isolate a single colony from a freshly streaked selective plate, and inoculate a culture of 1- 5 mL LB medium containing the appropriate selective antibiotic. Incubate for ~12-16 hours at 37°C with vigorous shaking (~ 300 rpm).

4.2.2. Centrifuge at 10,000 x g for 1 minute at room temperature.

4.2.3. Decant or aspirate and discard the culture media.

4.2.4. Add 250 µL Solution I/RNase A. Vortex or pipet up and down to mix thoroughly. Complete resuspension of cell pellet is vital for obtaining good yields.

Note: RNase A must be added to Solution I before use.

4.2.5. Transfer suspension into a new 1.5 mL microcentrifuge tube.

4.2.6. Add 250 µL Solution II. Invert and gently rotate the tube several times to obtain aclear lysate. A 2-3 minute incubation may be necessary.

Note: Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity. Do not allow the lysis reaction to proceed more than 5 minutes.

4.2.7. Add 350 µL Solution III. Immediately invert several times until a flocculent white precipitate forms.

Note: It is vital that the solution is mixed thoroughly and immediately after the addition of Solution III to avoid localized precipitation.

4.2.8. Centrifuge at maximum speed (≥13,000 x g) for 10 minutes. A compact white pellet will form. Promptly proceed to the next step.

4.2.9. Insert a DNA Mini Column into a 2 mL Collection Tube.

4.2.10. Transfer the cleared supernatant from Step 8 by carefully aspirating it into the DNA Mini Column. Be careful not to disturb the pellet and that no cellular debris is transferred to the DNA Mini Column.

4.2.11. Centrifuge at maximum speed for 1 minute.

4.2.12. Discard the filtrate and reuse the collection tube.

4.2.13. Add 500 µL HBC Buffer.

Note: HBC Buffer must be diluted with 100% isopropanol before use.

4.2.14. Centrifuge at maximum speed for 1 minute.

4.2.15. Discard the filtrate and reuse collection tube.

4.2.16. Add 700 µL DNA Wash Buffer.

Note: DNA Wash Buffer must be diluted with 100% ethanol prior to use.

4.2.17. Centrifuge at maximum speed for 1 minute.

4.2.18. Discard the filtrate and reuse the collection tube.

Optional: Repeat Steps 16-18 for a second DNA Wash Buffer wash step.

4.2.19. Centrifuge the empty DNA Mini Column for 2 minutes at maximum speed to dry the column matrix.

Note: It is important to dry the DNA Mini Column matrix before elution.

Residual ethanol may interfere with downstream applications.

4.2.20. Transfer the DNA Mini Column to a clean 1.5 mL microcentrifuge tube.

4.2.21. Add 30-100 µL Elution Buffer or sterile deionized water directly to the center of the column membrane.

Note: The efficiency of eluting DNA from the DNA Mini Column is dependent on PH. If using sterile deionized water, make sure that the pH is around 8.5.

4.2.22. Let sit at room temperature for 1 minute.

4.2.23. Centrifuge at maximum speed for 1 minute.

Note: This represents approximately 70% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration.

Page 8, Line 176-223.

4) 4.3: How is the enzyme digestion done? Cite a reference and mention enzyme concentrations.

A: Previous: 4.3 The above plasmids were verified by double restriction enzyme digestion using EcoR I and Bam H1.

Revised: 4.3 Verify the above plasmids using by double restriction enzyme digestion using EcoR I (15 U/μL) and Bam H1(15 U/μL). Page 8, Line 224-226.

5) Line 150: "Water bath at 37℃ for 2 hours" is incomplete

A: Previous: Water bath at 37℃ for 2 hours.

Revised: Incubate in water bath at 37℃ for 2 hours. Page9, Line 229.

3. Protocol Numbering: Add a one-line space between each protocol step.

A: A one-line space has been added between each protocol step. Please see

our manuscript for details.

4. Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.

2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

4) Notes cannot be filmed and should be excluded from highlighting.

A: The filmable contents used to make a video have been highlighted. Please see our manuscript for details.

5. Results: Please add at least one paragraph of results text that explains your representative results in the context of the technique you describe; i.e. how do these results show the technique, suggestions about how to analyze the outcome etc. This text should be written in paragraph form under a "Representative Results" heading and should refer to all of the results figures. You may include the figure captions under this heading but the captions and figure text must be separate entities.

A: In this protocol, porcine specific primers were designed, plasmids-containing porcine specific DNA fragments were constructed, and standard curves for quantitation were established. Species specificities of above 19 primers were confirmed by PCR. Species-specific primers (primer no.4 and primer no.11) were then used to quantify cpsDNA by qPCR in pig-to-mouse cell transplantation models and pig-to-monkey artery patch transplantation models[22].

Figure 1 Gene identity between pig and human or monkey.

Figure 2 Interspecies specificity and regular PCR detection of cpsDNA.

Figure 3 Establishment of standard curve for absolute quantification.

Figure 4 Key parameter Settings of qPCR software. Page12, Line 282-293.

6. Discussion: JoVE articles are focused on the methods and the

protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

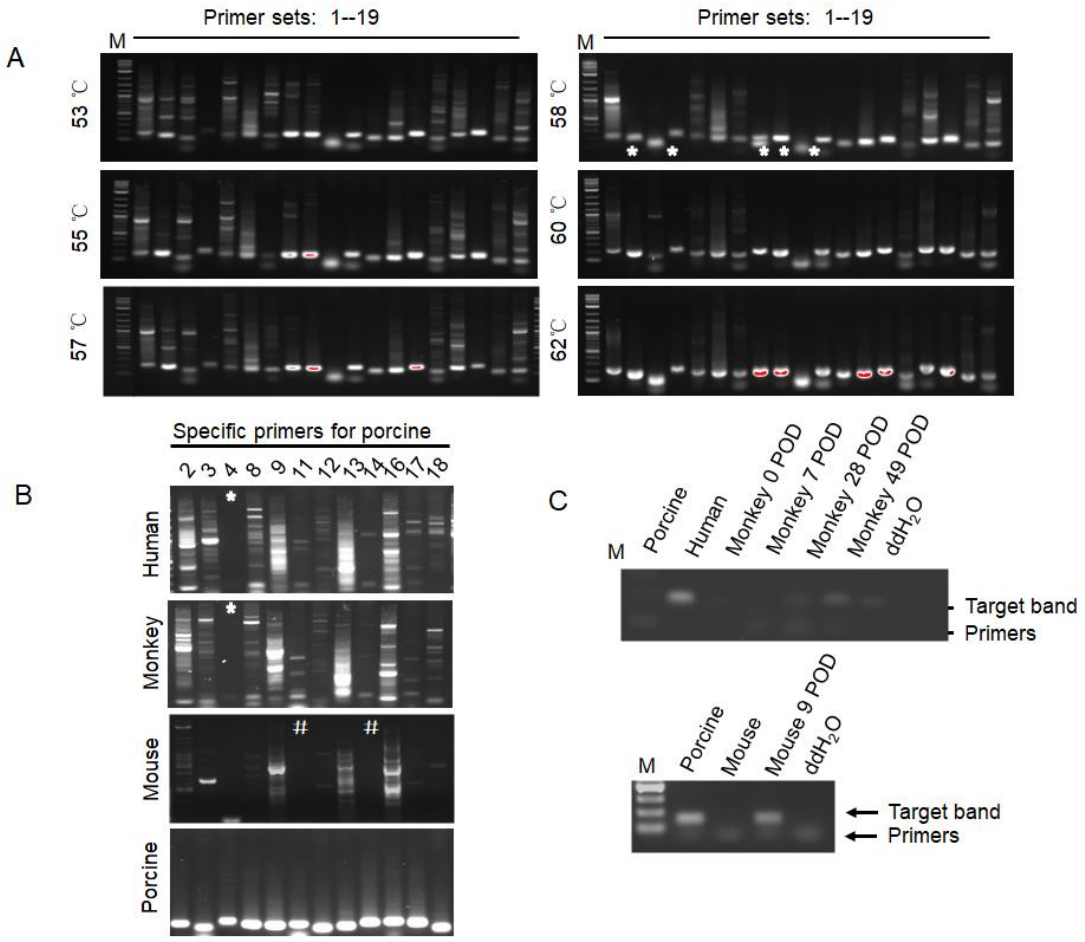
A: critical steps within the protocol: In our protocol, cfDNA should be extracted before qPCR. Will this cause cfDNA loss? The amount of DNA loss may be more than the amount of extracted DNA. Whether direct qPCR detection without DNA extraction is feasible requires further study. Page21, Line 381-383.

6. Figures/tables:

1) Your figure panel labeling scheme is confusing. Please use standard labels: A, B, C, etc.

2) Please remove the embedded figures/ tables from the manuscript. Figure legends, however, should remain within the manuscript text, directly below the Representative Results text.

A: Thank you for your suggestions. We revised it.



Avoid jpeg figures.

A: We have changed the style of our figures avoiding jpeg figures. Please see our manuscript for details.

Increase font sizes on fig 2.

A: We have increased font sizes on fig 2. Please see our manuscript for details.

6) It is unclear if there are 3 figures or 4 figures.

A: There are 4 figures in our manuscript. Please see our manuscript for details.

Page12, Line 287-293.

7. Figure/Table Legends: Please expand the legends to adequately describe the figures/tables. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.

A: **Figure 1 Gene identity between pig (sus scrofa, ssc) and human (homo sapiens, hsa) or monkey (Macaca fascicularis, mfa).** The gene sequences from three different species were compared by BLAST analysis. BLAST sequence analysis used genomic annotation information (the mRNA sequence) from the three species downloaded from NCBI (www.ncbi.nlm.nih.gov). The mRNA sequences of human, monkey, and pig genes were 139116, 65927, and 71498, respectively.

A: **Figure 2 Regular PCR validates specificity of porcine specific primers.** The pig genomic DNA fragments were amplified by primers 1-19 (**A**) . The human/monkey/mice genomic DNA fragment could not be amplified by some primers (primer no.4 and no.11) . The stars (*) indicate no amplifications in human/monkey genomic DNA. The pound sign (#) indicates no amplification in mouse genomic DNA (**B**). The two species-specificities primers (primer no.4 and no.11) were further proven to specifically amplify pig DNA in the pig-to-monkey artery patch and/or pig-to-mouse cell transplantation models, respectively (**C**).

A: **Figure 3 Establishment of the standard curve for absolute quantification.** The amplification plots, melt curve plots, and standard curve

views of (A) primer no.4 and (B) primer no.11 from the qPCR machine (see table of materials) are exhibited.

A: **Figure 4 The procedure of reaction system of qPCR was showed.** A good standard (R value close to 1, amplification efficiency is within 100%±5%) could be used up to half a year.

8. Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are The Beijing Genomics Institute, ExTaq, Gelred, Omega Biotech, THUNDERBIRD SYBR, Applied Biosystems (ViiA™7).

A: We have deleted all trademark, registered trademark symbols (TM/R) and the mention of company brand names.

9. Table of Materials: Sort the list alphabetically.

A: We have sorted list alphabetically and uploaded to the Editorial Manager site in the form of Excel files.

Previous：The value of this method suggests that it can be used as a simple,

convenient, cheap and less invasive method to monitor the immune rejection of xenotransplantation.

Revised：The value of this method suggests that it can be used as a simple, convenient, low cost, and less invasive method to monitor the immune rejection of xenotransplantation. Page 2, Line 47-49.

Reviewers' comments:

Reviewer #1:

Q: Minor edits: (1) "...artery patch transplantation models.Circulating DNA..." should be modified to "...artery patch transplantation models. Circulating DNA..."

A: Previous: This manuscript describes a simple and effective method to monitor immune rejection in pig-to-mouse cell transplantation models and pig-to-monkey artery patch transplantation models.Circulating DNA is a potentially non-invasive biomarker for organ damage. In this study, circulating pig-specific DNA (cpsDNA) was monitored during xenograft rejection by quantitative real-time PCR (qPCR).

Revised: This manuscript describes a simple and effective method to monitor immune rejection in pig-to-mouse cell transplantation models and pig-to-monkey artery patch transplantation models. Circulating DNA is a potentially non-invasive biomarker for organ damage. In this study, circulating pig-specific DNA (cpsDNA) was monitored during xenograft rejection by

quantitative real-time PCR (qPCR). Page 2, Line 39-43.

Q: Minor edits: (2) "And then using species-specific primers quantify..." should be modified to "Species-specific primers were then used to quantify.." or similar. This should be changed in the section titled "Summary" as well.

A: Previous：And then using species-specific primers quantify cpsDNA by qPCR in pig-to-mouse cell transplantation models and pig-to-monkey artery patch transplantation models.

Revised：Species-specific primers were then used to quantify cpsDNA by qPCR in pig-to-mouse cell transplantation models and pig-to-monkey artery patch transplantation models. Page 1-2, Line 46-47.

Q: Minor edits: (3) "Therefore, circulating porcine DNA be released into the recipient's blood post-xenotransplantation because of xeon-rejection" should be changed to "Therefore, circulating porcine DNA can be released into the recipient's blood post-xenotransplantation because of xeno-rejection".

A: Previous :Therefore, circulating porcine DNA be released into the recipient's blood post-xenotransplantation because of xeon-rejection.

Revised: Therefore, circulating porcine DNA can be released into the recipient's blood post-xenotransplantation because of xeno-rejection. Page 3,

Line 82-83.

Q: Minor edits: (4) "including blood from pigs, monkeys, volunteer humans, monkeys with pig grafts, and mice with porcine cells were extracted..." should be changed to "(including blood from pigs, monkeys, volunteer humans, monkeys with pig grafts, and mice with porcine cells) were extracted..."

A :Previous: The genomic DNA of the blood samples(including blood from pigs, monkeys, volunteer humans, monkeys with pig grafts and mice with porcine cells) were extracted using a genomic DNA Extraction Kit (Tiangen, DP304-02).

Revised: The genomic DNA (including blood from pigs, monkeys, volunteer humans, monkeys with pig grafts, and mice with porcine cells) were extracted using a genomic DNA Extraction Kit. Page 4, Line 107-110.

Q: Minor edits: (5) There are multiple extra spaces " " present that should be removed. Markup (underlining, highlighting) should be removed.

A: We have removed the multiple extra spaces " " and the markup. Please see our manuscript for details.

Reviewer #2:

Q: (1) Replace cheap with low cost

A: (1) Previous: The value of this method suggests that it can be used as a simple, convenient, cheap and less invasive method to monitor the immune rejection of xenotransplantation.

Revised: The value of this method suggests that it can be used as a simple, convenient, low cost, and less invasive method to monitor the immune rejection of xenotransplantation. Page 1, Line 32-44, Page 2, Line 47-49.

(2) Previous: This is less invasive, highly sensitive and specific, cheap, and time-saving.

Revised: This is less invasive, highly sensitive and specific, low cost, and time-saving. Page 3, Line 80-81.

Q: (2) Replace DNA maker with DNA marker

A: Previous: Add 5uL sample and 2-Log DNA Ladder (0.1–10.0 kb，NEB，N3200S) or DNA maker I (0.1–0.6 kb, TIANGEN, MD101-02) which contain 1uL 6×DNA loading buffer (Solarbio, D1010) into the Agarose gel，and then electrophorese at 120mA until the bands are separated.

Revised: Add 5μL sample and 2-Log DNA Ladder (0.1–10.0 kb) or DNA marker I (0.1–0.6 kb) which contain 1μL 6×DNA loading buffer into the Agarose gel, and then electrophorese at 120mA until the bands are separated. Page 8, Line 148-150.

Reviewer #3:

Q: (1) Add more specific information about the primers in Table 1. What's gene ID in NCBI_any link? What's genes and chromosomal locations for each primer? What's discrepancy of the primers with those from other species?

A: Specific information about the primers and Gene related information is shown in Table 1.

Q: (2) What kind of pig?

A: Information about the pig used in the experiment is in the material list.

Reviewer #4:

General remark

Q: (1) First, the authors use kits produced by Chinese companies without mentioning the kits during the working process. However this is necessary in order to know which solution has to be added. Also the columns used should be described in detail. The question is, whether all the materials from the Chinese companies are freely available everywhere.

A: The kits used in the study have described during the working process, and all the materials from the Chinese companies are freely available.

Q: (2) Second, some parts of the text which will be visualized are

confused, so there is the risk that the visualization will also be confuse.

A: We've corrected the confusing parts.

Q: (3) Third, the English of the text needs improvement, there are some typos (e.g., line 82 xeon-rejection).

A: We have corrected all the spelling mistakes. For example,

Previous：Therefore, circulating porcine DNA be released into the recipient's blood post-xenotransplantation because of xeon-rejection.

Revised: Therefore, circulating porcine DNA can be released into the recipient's blood post-xenotransplantation because of xeno-rejection. Page 3, Line 82-83.

Comments on specific points

Q: Line 98: the genes should also be different from mouse DNA

A: Previous：1.1 Whole-genome BLAST analysis was used to identify 19 porcine specific genes that were different from those of humans or monkeys, using Software of NCBI (www.ncbi.nlm.nih.gov).

Revised：1.1 Perform whole-genome BLAST analysis to identify porcine specific genes that were different from those of humans, monkeys or mouse, using Software of NCBI (www.ncbi.nlm.nih.gov). Page 4, Line 99-101.

Q: Line 11: what means beads?

A: Previous：Centrifugal briefly to remove beads from the inner wall of the tube covers. Add 500μL anhydrous ethyl alcohol and shake well.

Revised: Centrifuge briefly to the remove liquid beads from the inner wall of the tube covers. Add 500μL anhydrous ethyl alcohol and shake thoroughly. Page 5, Line 117-118.

Q: Line 129: predegeneration???

A: Previous：Put these 0.6mL EP tubes into PCR-Cycler，and run the program, perform the following steps：Predegeneration：95℃ for 30s; Denaturation 60℃ for 5s; Annealing 60℃ for 30s.

Revised: Put these 0.6mL EP tubes into the PCR-Cycler, perform the following steps: Denaturation: 95℃ for 5s; Annealing: 60℃ for 30s; extension:72℃ 30s.Page 5-6，Line 140-141.

Q: Line 144: In order to clone a fragment of pig DNA which than can be used for the generation of the standard curve, the primers used should be binding to this piece of pig DNA, so you need to define the fragment. This is extremely badly explained in this section.

A: What is unclear in this section has been redescribed in our manuscript

Q: Table 1: In context with the last remarks, the primers used and finally selected should be named indicating the gene and the Accession

number.

A: Primer no. 4 in human/monkey cohort (forward: 5′-TTCAATCCCA CTTCTTCCACCTAA-3′, reverse: 5′-CTTCATTCCATCTTCATAATAAC CCTGT-3′).

Primer no. 11 for mouse model (forward: 5′-TGCCGTGGTTTCC GTTGCTTG-3′, reverse: 5′-TCACATTTGATGGTCGTCTTGTCGTC T-3′).

Details of all the primers can be found in Table1. Page 7, Line 169-175.

Q: Line 155: particles??

A: Previous：Number of particles in 1mL starting standard solution：N= (2×10E11)/ (6.02×10E23) mol;

Revised: Number of plasmid in 1mL starting standard solution：N= (2×10E11)/ (6.02×10E23) mol; Page 9, Line 235-236.

Q: Line 165: dark environment???

A: Previous：Prepare reaction system of qPCR（all steps are performed in a dark environment）: Add the pre-mixed solution which contains 325μL THUNDERBIRD SYBR qPCR Mix, 13μL 5' primer, 13 μL 3' primer, and 169μL ddH2O into a 1.5mL EP tube, which was then thoroughly mixed and slightly centrifuged.

Revised: Prepare reaction system of qPCR（all steps are protected from

light) : Add the pre-mixed solution which contains 325μL qPCR Mix, 13μL 5' primer, 13 μL 3' primer, and 169μL ddH2O into a 1.5mL EP tube, which was then thoroughly mixed and slightly centrifuged. Page 9, Line 245-248.

Q: Figure 1: It is absolutely unclear what is shown in Figure 1A, B1-B3. Please explain it much better.

A: The pig genomic DNA fragments were amplified by primers 1-19 (A) . The human/monkey/mice genomic DNA fragment could not be amplified by some primers(primer no.4 and no.11). The stars (*) indicate no amplifications in human/monkey genomic DNA. The pound sign (#) indicates no amplification in mouse genomic DNA (B). The two species-specificities primers (primer no.4 and no.11) were further proven to specifically amplify pig DNA in the pig-to-monkey artery patch and/or pig-to-mouse cell transplantation models, respectively (C). Page 14, Line 312-319.

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