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Isolation of Circulating Fetal Trophoblasts by a Four-Stage Inertial Microfluidic Device for Single-Cell Analysis and Noninvasive Prenatal Testing

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Experimental Section

Chip design and fabrication

In practical, the CelutriateChip 1 has four stages; as waste subchannel and buffer subchannel are not necessary for the first and last stage, these two stages only have two subchannels accordingly. Each stage has about two loops of spirals, and the elutriation subchannels are 160 μm in depth and 800-1000 μm in width. The buffer and waste subchannels are designed at each downstream stage, about 70% of the volume in its elutriation subchannels shift to its waste subchannel and replenish with a fresh buffer from its upstream buffer subchannel. As shown in **Figure 1C**, the radius of curvature (RoC) increases dramatically from the inner loops to the outer loops of the spiral. There is an inverse correlation between Dean flow and the channel RoC, which means it requires a long travelling channel to move the unwanted cells to the outside of the elutriation subchannel in a large RoC spiral stage. For a channel with a constant cross-section and flow rate, a longer channel also means a higher pressure drop. If we put all the stages in one coil, the radius of the outer loops' curvature must be large enough to accommodate those loops inside. Therefore, we divide the four stages into two coils to reduce the channel length, as well as the pressure drop of the channel in the design. Stage 1 and 2 are put in the first coil, stage 3 and 4 are put in the second coil, and a straight channel section is used to connect the two coils. Besides the spirals, the chip also has one buffer channel and one sample channel as inlets to bring in buffer and sample accordingly, and one recovery channel and one waste channel as outlets to lead out the recovery and waste cells. At the open end of each inlet and outlet channels, there is a port to connect the chip to a flow control system so that the fluid in each inlet or outlet channels has a stable given flow rate. The CelutriateChip 1 is fabricated with plastics, provided by Shenzhen Genflow Technologies Co., Ltd., China.

Flow rate optimization

Pre-diluted human peripheral blood is used to find out the optimized total flow rate and sample throughput of the chip. A pair of LSP01-1BH syringe pumps are used (Longer Precision Pump Co., Ltd., Hebei, China) to dispense sample and buffer separately. The flow rate calibration is taken under Olympus CKX53

inverted microscope at phase-contrast mode. For each video, 10 frames are stacked together to enhance the image of moving cells to show the quality of the blood depletion and target cell focusing. The flow rates are adjusted during the calibration until the optimized total flow rate and maximum throughput are found. After optimized, no blood cells are observed in the recovery output channel. After the confirmation of the total flow rate, A typical cell line with a similar range of trophoblastic cell size, A549, is diluted to ~2000 cells/mL and used to evaluate the cell focusing quality at the same flow rate configuration. Microscope videos at each stage junctions and output bifurcation are taken to check if the cells are focused at the end of each stage and whether the major cell stream jumps into the elutriation subchannel of the next stage or the output channel.

Recovery evaluation

To further automate the separation process, the CelutriateChip 1 is integrated to a flow control platform, Celutriator TX1 (Shenzhen Genflow Technologies Co., Ltd., China), for a stable and continuous dispense of sample and buffer to the CelutriateChip 1, as well as an automatic collection of the enriched suspension (**Figure S1**). Pre-labeled and counted trophoblastic cells are spiked into the donor's peripheral blood and processed with Celutriator TX1 and recovered in a 10 mL centrifuge tube. The recovery volume is about 1:1 of the sample volume. To evaluate the cell number, the recovery was centrifuged at 2500 rpm for 20 min, re-suspended to 200 μL, transferred to a 96 well plate, left for 20 min and counted under an inverted fluorescent microscope (Olympus CKX41).

Cell culture and sample preparation

A549, HTR8-S/Vneo, JEG-3 and JAR cell lines were purchased from the American Type Culture Collection and used to mimic the CTBs separation. Cells were cultured by using a standard culture protocol, as described previously.[1] After that, trophoblastic cells were collected and pre-labelled with CFDA-SE (Keygen Biotech, Jiang Su, China). Artificial samples were prepared by spiking trophoblastic cells into the non-pregnant donor's blood. These samples were processed by CelutriateChip 1 and the collected CTBs at the outlet were used for further analysis.

Single-cell isolation and immunofluorescence staining

Cells at the suspension collected from the outlet were harvested by centrifugation at 2500 rpm for 20 min and re-suspended in 200 μL of PBS. Individual CTBs were picked using a 40 μm micropipette, as described in our previous study.[1] These cells were layered on a slide and dried at 56 °C for 30 min. Subsequently, the cells were fixed with 4% paraformaldehyde for 20 min, permeabilized by 0.15% TritonX-100 for 30 min, and blocked with 2% BSA for 30 min at room temperature. The cells were then incubated with PE-conjugated CK-7 antibody (1:100) (Bioss, Beijing, China) and FITC-conjugated CD45 antibody (1:100) (Bioss, Beijing, China) overnight at 4 °C. After that, the cells were stained with DAPI (Keygen Biotech, Jiang Su, China) for 15 min and imaged by an Olympus BX51 (Tokyo, Japan) fluorescence microscope.

Whole Genome Amplification (WGA)

Individual target cells were transferred into PCR tubes and amplified by REPLI-g Single Cell Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, PCR tubes containing the target cells were added with 3 μL of denaturation buffer and incubated at 65 °C for 10 min, then treated with 3 μL of stop solution and 40 μL of master mix and reacted at 30 °C for 9 h.

Quality assessment for WGA products

The concentration and size of DNA amplified from CTBs were evaluated by the Agilent 2100 bioanalyzer system (Agilent Technologies, Waldbronn, Germany). 6 μL of WGA product was loaded on DNA 1000 Lab Chip and the chip was mixed by a Vortex Mixer at 2400 g for 1 min. The chip was processed by Agilent 2100 bioanalyzer immediately. Moreover, the DNA quality was also assessed by PCR amplification of 22 SNP loci across 22 chromosomes. The PCR conditions were the same as previously described.[1] The PCR products were directly analyzed by 2% agarose gel electrophoresis. The gel was stained with 1×4S red plus nucleic acid stain (Sangon Biotechnology Co. Ltd., Shanghai, China) and imaged using the Gel Doc XR+ system (Bio-Rad, USA).

STR and SNP analysis

Fragments for 21 human STR loci were amplified using a PowerPlex System according to the manufacturer's instructions. The multiplex PCR products were loaded and detected by an ABI3130xl Genetic Analyzer (Applied Biosystems Inc., Massachusetts, USA). The results were analyzed using GeneMapper 4.2 Software (Applied Biosystems Inc., Massachusetts, USA).

For SNP analysis, the genotype of 22 SNP loci from 22 chromosomes was determined by Sanger sequencing. Briefly, amplifications were performed in a Mastercycler® X50s PCR thermocycler (Eppendorf, Hamburg, Germany) with 100 ng of WGA DNA as a template. Touchdown PCR was applied to amplify the target fragments (Table S2). The PCR products were evaluated by 2% agarose gel electrophoresis and directly sequenced using an ABI Genetic Analyzer 3730xl (Applied Biosystems Inc., Massachusetts, USA).

Clinical samples

Whole blood samples were collected from Nanfang Hospital, Southern Medical University (Guangzhou, China), including 30 from pregnancies and 10 from non-pregnant women. The present study was approved by the Ethics Committee of Nanfang Hospital, Southern Medical University. Prior informed consent was obtained from all subjects. Blood samples were collected in EDTA-K3 tubes and processed within 2 h. 2 mL of whole blood was processed on CelutriateChip 1 and the enriched suspension was used for downstream analysis. The enriched cells were used for immunostaining of CK-7 and CD45, and individual CK-7^{+}/$CD45^{-}$/$DAPI^{+}$ cells were isolated for WGA. WGA product was used as a template to amplify target genes. The PCR products were analyzed by Sanger sequencing.

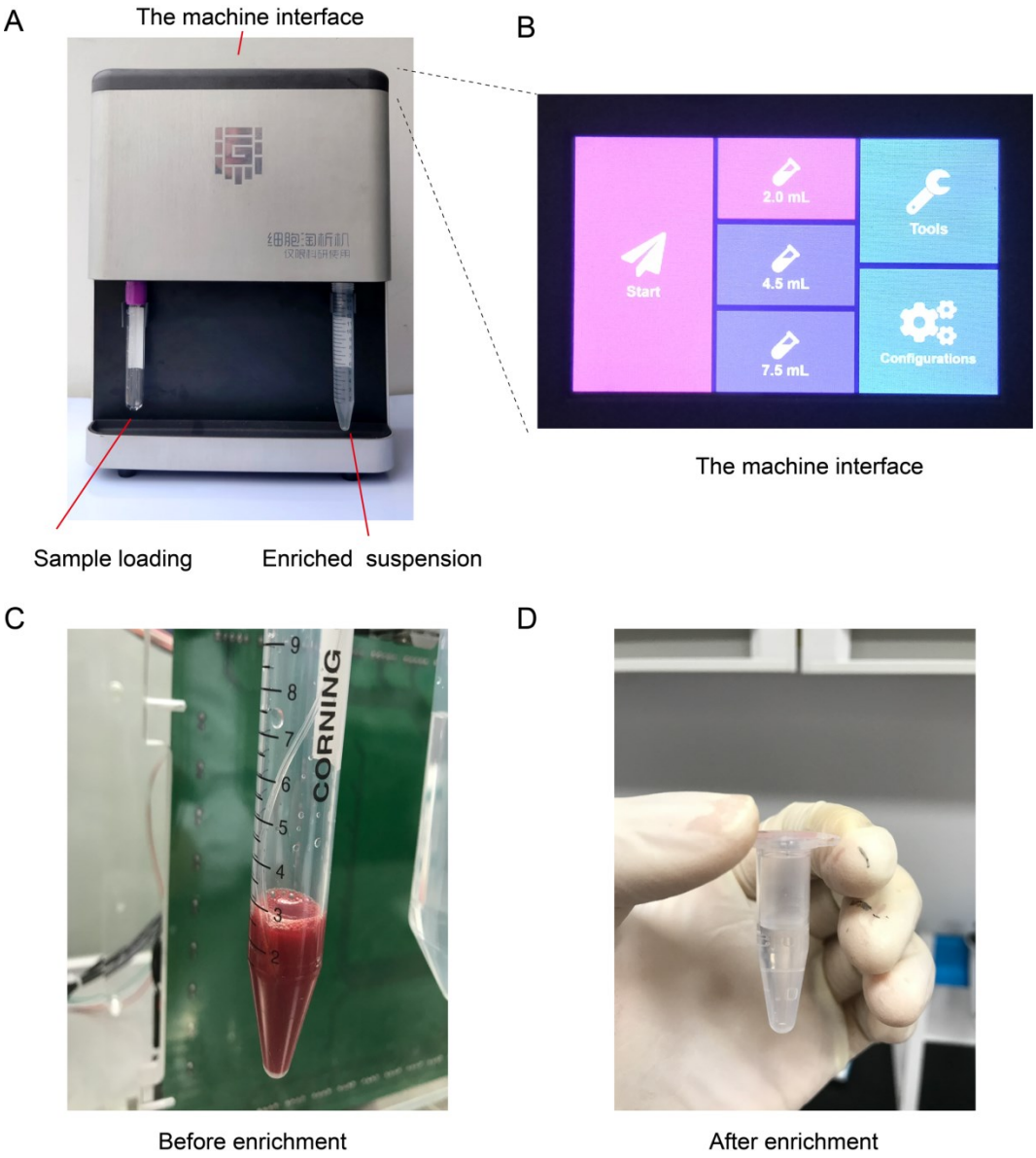

Figure S1 The Celutriator TX1 microfluidic device. (A) Practicality picture. (B) The machine interface. (C) Before enrichment. (D) After enrichment.

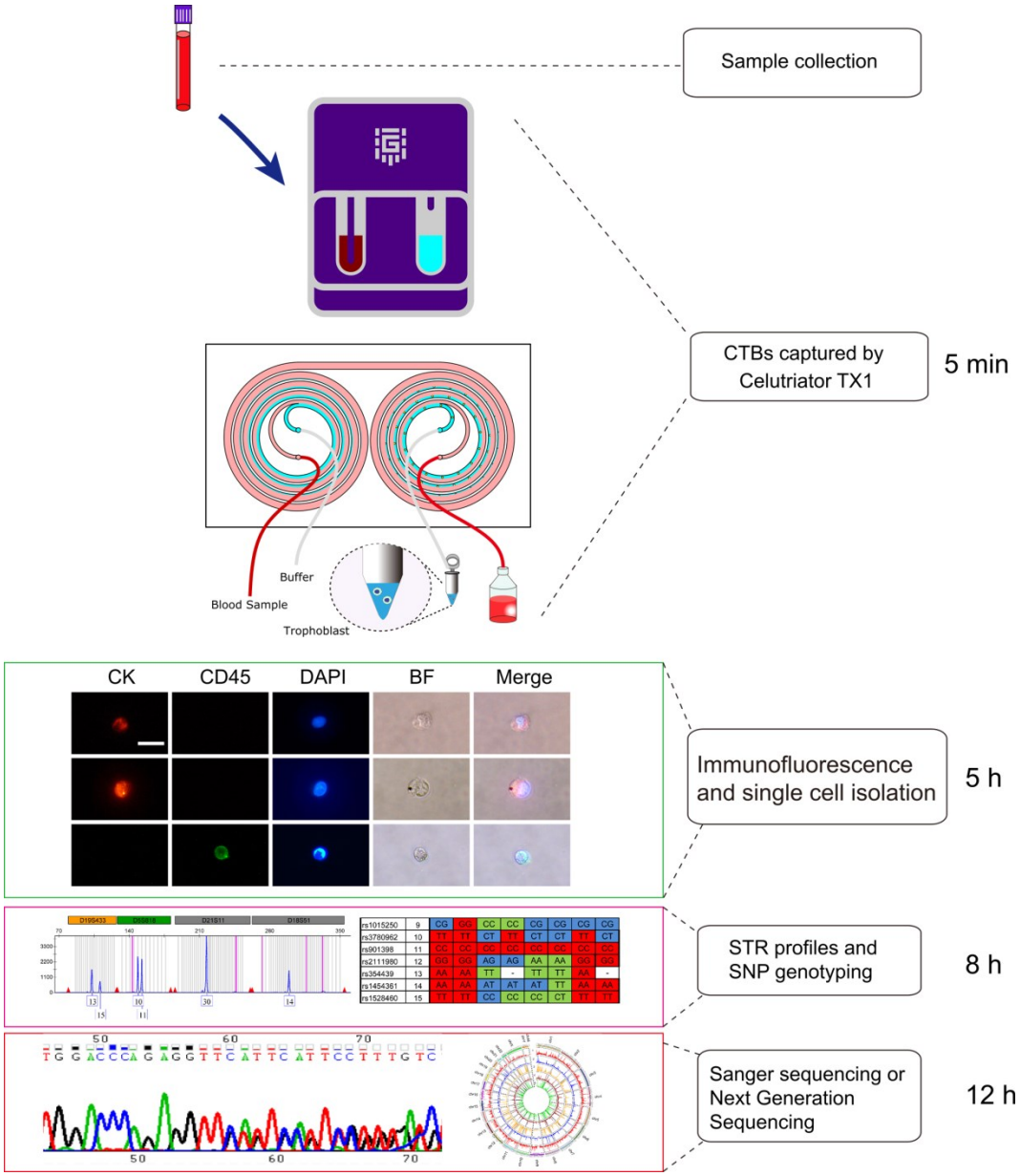

Figure S2 The workflow and assay time for analyzing circulating trophoblastic cells by the CelutriateChip 1 system.

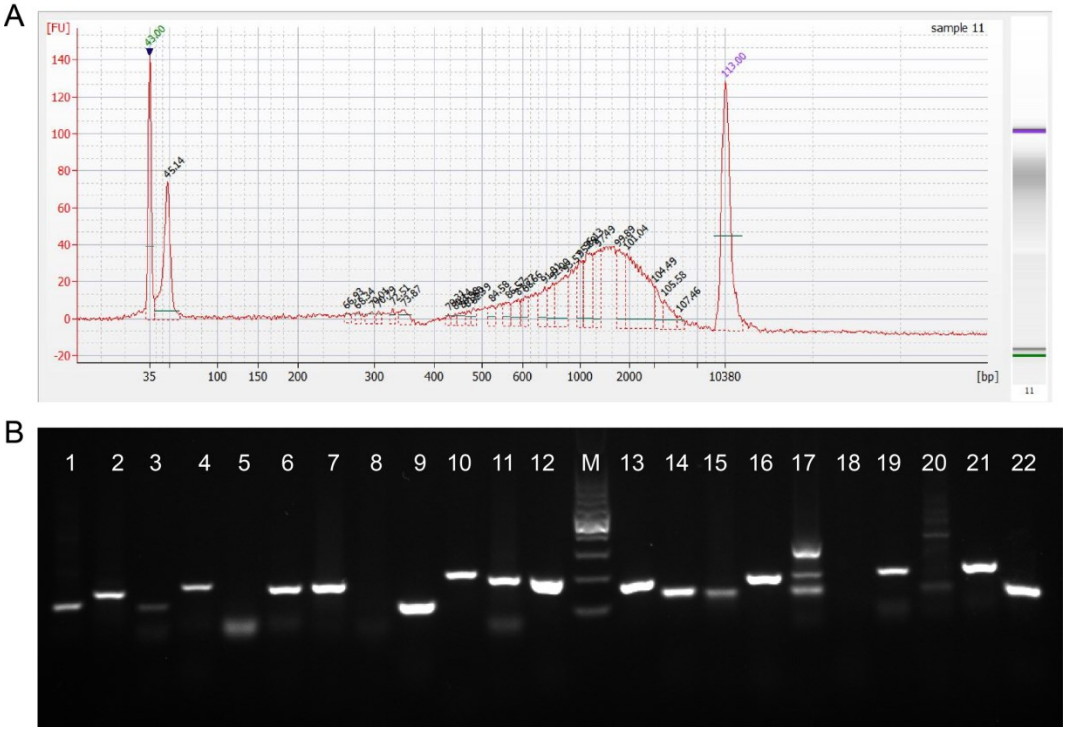

Figure S3 The concentration and integrity of WGA products of CTBs isolated from clinical samples are evaluated by Agilent 2100 bioanalyzer. (A) Representative results show that the average concentration of WGA products is 779.5 ng/μL and the DNA signal peak range from 400 to 5,000 bp. Furthermore, PCR reactions of 22 genetic loci are also performed (B). We found that about 18/22 (81.8%) of genetic loci are detectable.

Table S1. Clinicopathological characteristics for a total of 30 pregnant subjects.

Patient ID	Age (years)	Gestational weeks	Gravidity	Parity	CTBs / 2 mL blood
P1	28	30	2	0	1
P2	30	37	1	0	2
P3	35	36	2	0	2
P4	24	29	2	1	2
P6	27	32	1	0	1
P8	27	24	2	1	2
P9	34	29	3	1	2
P10	27	31	2	1	1
P11	28	12	3	2	1
P12	28	11	1	0	3
P14	31	40	2	0	1
P15	29	26	2	0	2
P18	25	12	1	0	2
P19	29	28	1	0	1
P20	32	37	4	1	0
P21	20	36	2	1	2
P22	38	38	2	1	3
P23	25	36	1	0	0
P24	21	18	1	0	3
P26	27	30	1	0	1
P27	25	30	3	1	0
P28	27	37	3	1	1
P29	21	23	1	0	2
P31	27	39	1	0	1
P32	22	8	1	0	1
P33	32	39	2	1	3
P34	25	37	2	0	2
P35	24	37	2	0	2
P36	29	37	3	1	3
P37	33	38	3	1	0

Table S2. List of 22 SNPs and their sequencing primers.

SNPs	Chromosome		Sequence (5'-3')	Size	Annealing temperature(℃)
rs1413212	1	F	CAAAAGATTGGACAGGCTAACAGG	138 bp	touchdown PCR： 95℃ 5min 94℃ 30s 64℃30s for 2 cycles 63℃30s for 2 cycles 62℃30s for 2 cycles 61℃30s for 2 cycles 60℃30s for 2 cycles 59℃30s for 2 cycles 58℃30s for 2 cycles 57℃30s for 2 cycles 56℃30s for 2 cycles 55℃30s for 2 cycles 54℃30s for 15 cycles 72℃ 1min
		R	GGTCAACAACCTCCTTTGGAAACA		
rs1109037	2	F	TGTCACAGAGCTGGTGGTGA	178 bp	
		R	GGCTGAAAGATGATGGCAGAG		
rs4364205	3	F	TTCCAGTCCTAGATATCCACCCAT	125 bp	
		R	TTCACCATTTGATAGCCATTTGG		
rs1979255	4	F	CATGGGGGTTTTTGAAGACTTTCG	202 bp	
		R	GGATGGTATTTAGGTCAAATGAATTTCCA		
rs338882	5	F	TTCTCTACCAGCTGTGCTCGTG	174 bp	
		R	GCTTCATTTTTCTCTCCTTCTGTCTC		
rs13218440	6	F	CTGCTGTGGACTGAAACTTGATC	185 bp	
		R	CTACCAATGTGCTACGTACCCTAC		
rs917118	7	F	CCAACAGAGTCCTCCATGAAGAT	193 bp	
		R	GAGCTCAGAAAGGTGAGTGAGG		
rs4606077	8	F	GTCGGGAATCTGAGCTCG	169 bp	
		R	TGTGGCTTTGTAGTTCTAGTGTG		
rs1015250	9	F	AGGGTAAAAGGTTACTAAGTGATGGAGT	114 bp	
		R	CATAAGACATTAGGTGGATTCATAGCTGTTTA		
rs3780962	10	F	GCCACTGAAAGTGATGGC	223 bp	
		R	TGCGGTAGCGGGCTTTT		
rs901398	11	F	CTGGGTGCAAACTAGCTGAATATC	202 bp	
		R	GCTGATGATGGGCCTGG		
rs2111980	12	F	GAGAACTCCAGGCTGTGACTAAGTC	202 bp	
		R	GCCATGTTGTAAACATTTTTACGGTCAA		
rs354439	13	F	GGCTTCTCTTTCCCTTATGTATCTC	171 bp	
		R	GCTTGACAGTTTGCCTGC		
rs1454361	14	F	TGTCCATCATCAGTAAGACACTTTTCAG	126 bp	
		R	AAAACCACCATCTCCAGCAAGT		
rs1528460	15	F	TCCTGGAGATCAATATTTAGCCTTA	115 bp	
		R	GGGTGACCAGTAGTTCTATGAGC		
rs1382387	16	F	TGTCTCCATGTGCACAAGGAGC	184 bp	
		R	TCATTCCCATGTTGTGTACACGAAA		
rs2292972	17	F	CCCTGCCGGGTCACGAG	187 bp	
		R	TGAATGAGGGACTGGCACCTG		
rs1493232	18	F	TCTCAAGGAATTAATCACCAAAGCTATTCT	206 bp	
		R	AAATGGTACTGTGGAAAGCAGCATAA		
rs576261	19	F	CTCCCCCTCCGTGTACCA	192 bp	
		R	CAGAATTCAGGGACCAAACTCC		
rs1523537	20	F	GCTTAGGGTCTTAATACATTCATTTCTGC	120 bp	
		R	ACTGGGTGAGACAATGCACA		
rs221956	21	F	ATGAATGCTTTCCCTCCAGCTC	213 bp	
		R	TGACTGAGACAGAAAGTGGCCC		
rs2040411	22	F	ATGAATGCTTTCCCTCCAGCTC	103 bp	
		R	GGGTCCATGCTAGAAAAAGCTG		

Primer Name	Sequence (5'-3')	Size	Annealing temperature(℃)
HBB1	F AGAAGAGCCAAGGACAGGTACG R CTCTGTCTCCACATGCCCAGTT	353 bp	60
HBB2	F TCTGATAGGCACTGACTCTCTCTG R GGGGAAAGAAAACATCAAGCG	309 bp	62
HBB3	F AAGTCAGGGCAGAGCCATCTA R GTGCCTATCAGAAACCCAAGAGT	260 bp	60
HBB4	F GATAAGGCTGGATTATTCTGAG R ACTTAGGGAACAAAGGAACC	258 bp	60

Table S3. Primers used for the amplification of *HBB* gene.

Notes and references

1. Y. Huang, B. Situ, L. Huang, Y. Cao, H. Sui, X. Ye, X. Jiang, A. Liang, M. Tao, S. Luo, Y. Zhang, M. Zhong and L. Zheng, Adv Sci (Weinh), 2020, 7, 1903354.