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sQuantGenome ver. 1.1: Streamlined protocol for quantitative PCR-based genome size estimation

The protocol allows genome size estimation without requiring any live cells or materials for reference species with known genome sizes. It is based on the method reported by Wilhelm *et al.*[1] and also incorporates several additional steps to improve the estimation accuracy. The significant modifications are 1) selection of multiple (at least three) genes from the established single-copy orthologue gene set CVG[2], 2) use of the sheared gDNA instead of HMW gDNA for DNA quantification and PCR, 3) careful evaluation of the DNA copy number standards by Sanger sequencing and size distribution analysis by 2100 Bioanalyzer, 4) use of low-bind plasticware (tubes and pipette tips) and a buffer that contains a surfactant (Tween-20) to prevent the adsorption of DNA to the plastic surface.

The workflow consists of high molecular weight (HMW) genomic DNA extraction and DNA shearing, identification of single-copy gene transcripts from the database and primer design, preparation of qPCR copy number standards by PCR, and quantitative PCR (Figure 1).

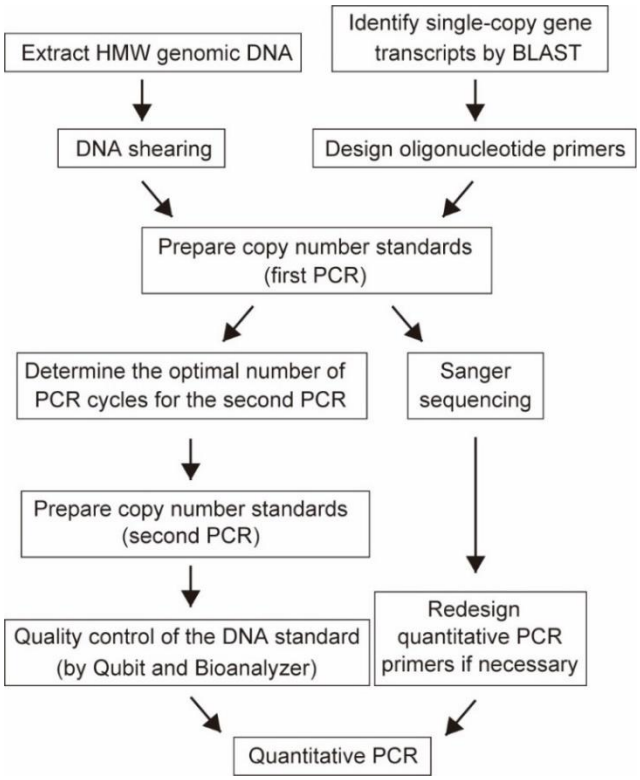


Figure 1. Workflow of the genome size estimation by quantitative PCR

Reagents and consumables

- ✓ Low retention tubes and pipet tips: DNA LoBind tube (1.5 mL, 2.0 mL; Eppendorf), Sapphire Low-Retention Pipette Tip (1250 µL, 300 µL, 20 µL; Greiner Bio-one), Neptune S3 Low Retention Pipet Tip (100 µL; Neptune Scientific), and ART Low-Retention Pipette Tip (10 µL; ThermoFisher Scientific).
- ✓ Micro pipettes: Pipetman (P1000, P200, P20, P10, P2; Gilson), and Picus 8-channel electronic pipette (0.2-10 µL; Sartorius).
- ✓ Motar and pestle (for gDNA extraction from tissue)
- ✓ Liquid nitrogen (for gDNA extraction from tissue)
- ✓ PBS (10 × PBS solution, Nippon Gene, cat: 314-90185)
- ✓ Shark phosphate-buffered saline solution (for gDNA extraction from elasmobranch red blood cells; shark PBS: 1× PBS supplemented with 299 mM urea and 68 mM NaCl) for elasmobranch cells/tissue[3,4]. Dissolve 4.49 g of urea and 1.0 g of NaCl in 200 mL of distilled H2O. Add 25 mL of 10× PBS. Adjust pH to 7.0 with 1N HCl. Adjust the volume to 250 mL with H2O. Sterilize by filtration and store at 4°C.
- ✓ NucleoBond AXG 100 or AXG 500 with NucleoBond Buffer Set IV (Clontech) or DNeasy Blood and Tissue kit (Qiagen).
- ✓ TE buffer
- ✓ Nuclease-Free Water (Qiagen)
- ✓ g-TUBE (Covaris)
- ✓ TapeStation Genomic DNA ScreenTape analysis kit (Agilent)
- ✓ KAPA HiFi HotStart ReadyMix (KAPA Biosystems)
- ✓ 8-well PCR tube (BIO-BIK)
- ✓ Agarose S gel (Nippon gene)
- ✓ 10× TBE (Tris-borate-EDTA) buffer
- ✓ GelStar (Lonza)
- ✓ AMPure XP beads (Beckman Coulter)
- ✓ MagnaStand v3.2 (FastGene)
- ✓ Qubit dsDNA HS Assay Kit (ThermoFisher Scientific)
- ✓ BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific)
- ✓ QuantiTect SYBR Green PCR Kit (Qiagen)
- ✓ Bioanalyzer High Sensitivity DNA Kit (Agilent)
- ✓ 50× dilution buffer (DB) (500 mM Tris-HCl pH 8.0, 2.5% Tween-20)
- ✓ 96-well and 384-well PCR plates (Eppendorf)

Genomic DNA extraction

1. Preparation for animal tissue/embryo samples.
    1.1 Dissect tissue into small pieces (up to 1 cm^3 in size), flash freeze in liquid nitrogen, and store at -80°C until use.
    1.2 Powderize the animal tissue/embryo in liquid nitrogen using a mortar and a pestle.
        1.2.1 Pre-cool a mortar and a pestle in an ultra-low temperature freezer for at least 1 hr.
        1.2.2 Pour liquid nitrogen into the pre-cooled mortar with a pestle.
        1.2.3 Transfer tissue (up to 1 cm^3 in size) into the mortar and grind with the pestle until it becomes a fine powder.
        Note: Pour liquid nitrogen into the mortar occasionally to keep the motar and the pestle cold during the entire grinding process.
        1.2.4 Transfer the tissue suspension, in liquid nitrogen, into a 50 mL tube pre-cooled with liquid nitrogen.
        1.2.5 Close the screw cap loosely, put the tube in an ultra-low temperature freezer, and wait until the liquid nitrogen evaporates completely.
        1.2.6 Proceed immediately to genomic DNA (gDNA) extraction or store the powderized tissue at -80°C until use.
2. Preparation for red blood cell samples.
    2.1 Draw blood from the animal using a syringe containing heparin sodium salt solution (MOCHIDA, cat: 5,000 units/5 mL; use 10 µL for 2-3 mL of blood) or EDTA (0.5M EDTA, Gibco, cat: 15575-020; use 10 µL for 1-2 mL of blood).
    2.2 Dilute the blood 1:200 with PBS (1 × PBS or shark PBS) and count cell number using the disposable cell counting plate.
    Note: Use shark PBS for elasmobranchs (sharks and rays).
    2.3 Take 1 × 10^6 – 1 × 10^7 cells in a 1.5 mL microtube, pellet the cells at 500 ×g, 5 min, 4°C, remove supernatant, flash freeze in liquid nitrogen, and store at -80°C until use.
3. Extract high molecular gDNA using NucleoBond AXG 100 or AXG 500 with NucleoBond Buffer Set IV (Clontech) or DNeasy Blood and Tissue kit (Qiagen).
    Note: The former kit was used only when the extracted gDNA is used for other purposes that particularly require high molecular weight DNA such as long-read genome sequencing.
4. Quantify the concentration of the gDNA by Qubit using the dsDNA HS Kit.
5. Analyze the size distribution of the extracted gDNA using 4200 TapeStation (Agilent Technologies) with the Genomic DNA ScreenTape analysis kit and confirm the main band to be over 10 kb.

6. Take 1-4 µg of gDNA, adjust the total volume to 150 µL with TE buffer, and apply to the g-TUBE column (Covaris). Shear the gDNA to 10 kb at 6,100 rpm for 1 min, 25°C, in an MX-300 high-speed centrifuge (TOMY) equipped with an AR015-24 rotor (TOMY).
   Note: Contact Covaris Inc. to obtain information of centrifuge speed (rpm) for other rotor types.
7. Take 1 µL of the sheared gDNA and analyze the size distribution using 4200 TapeStation with the Genomic DNA ScreenTape analysis kit. Confirm the size of the main band to be around 10 kb.
8. Store the sheared gDNA at 4°C.

Selection of reference genes and primer design

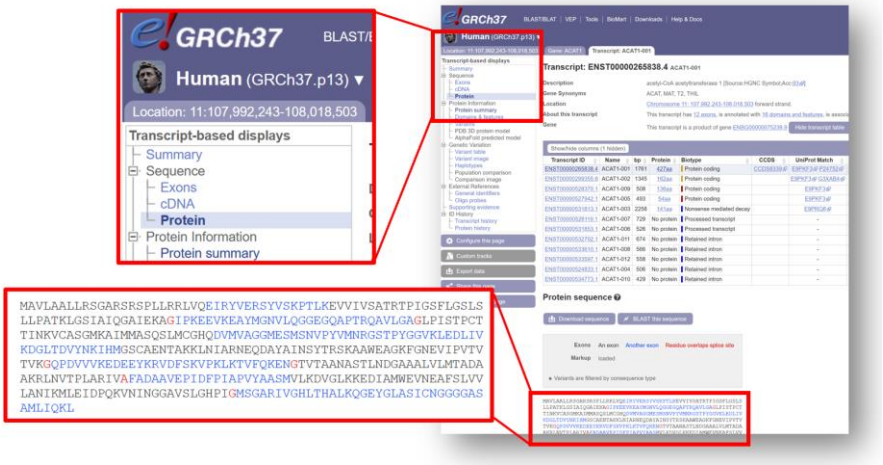
1. Fifteen Core Vertebrate Genes (CVG)[2] that are also included in the Core Eukaryotic Genes (CEG)[5] and the vertebrate gene set of BUSCO (Benchmarking Universal Single-Copy Orthologs)[6] identified by Hara *et al*.[2] were used for the analysis.

HGNC symbol	Human peptide ID
ACAT1	ENSP00000265838
ADCK1	ENSP00000238561
CCT4	ENSP00000377958
COQ5	ENSP00000288532
DLD	ENSP00000205402
GUF1	ENSP00000281543
HACL1	ENSP00000323811
MPI	ENSP00000318318
MTIF2	ENSP00000263629
NAT10	ENSP00000257829
POLR3H	ENSP00000347345
PSMD6	ENSP00000295901
RFC3	ENSP00000369411
RFC4	ENSP00000296273
ZNF622	ENSP00000310042

2. Identify the target species' candidate CVG transcript by performing BLAST (TBLASTN) search using the human CVG protein sequence shown above as a query, against the target species' transcriptome assembly, e.g., Squalomix (https://github.com/Squalomix/info)[7] for elasmobranch species.

Note: For species other than those included in Squalomix, perform BLAST (TBLASTN) search against the species' transcript database.

3. Obtain the deduced amino acid sequence of the best hit transcript, and use it as a query in BLAST (BLASTP) search against the human protein database. The target species transcript displaying reciprocal best hits in the TBLASTN and BLASTP searches was considered orthologous to the selected CVG.
   Note: It will also be ideal if the orthology is confirmed with molecular phylogeny inference.
4. Identify the putative last exon of the target species' CVG transcript by aligning the target species' transcript sequence to the last exon of the human counterpart.
   Note: The last exon was chosen because it is usually the longest in the eukaryote genome[8,9]. It is also based on the assumption that, the exon-intron structure is conserved, for example, between elasmobranchs and mammals. Putative positions of introns for a certain transcript of a selected gene are clearly instructed by the 'Protein' view at Ensembl (see below, for an example).



5. Design qPCR primers (inner primers) inside the putative last exon of the CVG transcript, e.g., using the Primer Express software (ThermoFisher Scientific).
   Note: For species with known gene structure, simply design qPCR primers (inner primers) and PCR primers (outer primers) inside the last exon of the transcript.
6. Design PCR primers (outer primers) outside the qPCR target region but within the putative last exon of the CVG transcript that generates amplicon of 130-300 nt in size using a reliable primer design program such as Primer3 (https://bioinfo.ut.ee/primer3/)[10].
   Note: Design primers for at least 5 CVG genes as some of them may fail to work.
7. Calculate molecular weight (MW) of the DNA standards to be amplified with the outer primers by the OligoCalc program (http://biotools.nubic.northwestern.edu/OligoCalc.html).
   Note: Oligonucleotide composition should be set to "double-stranded DNA".

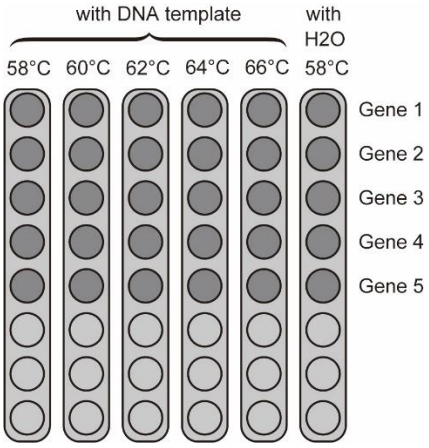
Preparation of qPCR copy number standards

First PCR

1. Dilute the g-TUBE-sheared gDNA to 5 ng/μL with H2O.
   Note: Prepare enough DNA for the first PCR.
2. Prepare PCR master mix.

PCR Master Mix (First-PCR-mix)	(×1 reaction)	(# of temperature gradient +NTC)	(10% extra)		
2× KAPA HiFi HotStart Ready Mix	10 μL	×6	×1.1	=	66 μL
Forward primer (10 uM)	0.6 μL	×6	×1.1	=	3.96 μL
Reverse primer (10 uM)	0.6 μL	×6	×1.1	=	3.96 μL
H2O	7.8 μL	×6	×1.1	=	51.48 μL
Total	19 μL	×6	×1.1	=	125.4 μL

3. Dispense 19 μL of the First-PCR-mix in 8-well PCR tubes as shown below and add 1 μL of diluted template DNA (at 5 ng/μL) or H2O.



   Note: The layout shown above is for five CVG genes and the annealing temperature gradient of 58°C - 66°C in 2°C increments. Modify the layout for the different number of CVG genes or different temperature gradients.
4. Perform PCR at [98°C for 3 min, 34 cycles of (98°C for 20 sec, 58°C - 66°C in 2°C increments of temperature gradient for 15 sec, 72°C for 15 sec), 72°C for 1 min, and a hold at 4°C].
   Note: Run PCR separately for each annealing temperature condition or use a PCR machine equipped with a temperature gradient protocol.
5. Take a portion (4 μL) of the PCR product, run electrophoresis with a 2% agarose (Agarose S) gel in 1× TBE buffer, stain the gel with GelStar and determine the optimal PCR condition that gives a single-band or near single-band product.
6. Take 15 μL of the PCR product from the optimal PCR condition and add 5 μL of TE.
7. Double size-select the PCR product using AMPure XP beads as follows.

PCR product size range (bp)	Size selection condition (right side – left side)
100-160	×0.8 vol. (16 µL AMPure XP) – ×1.8 vol. (20 µL AMPure XP)
160-240	×0.8 vol. (16 µL AMPure XP) – ×1.2 vol. (8 µL AMPure XP)
240-300	×0.7 vol. (14 µL AMPure XP) – ×1.0 vol. (6 µL AMPure XP)

Right-side selection

7.1 Add AMPure XP beads (for right-side selection; 16 µL or 14 µL) to the 20 µL PCR product, vortex mix, and incubate 5 min at room temperature.

7.2 Quick spin the tube, put on the magnet, and wait until the supernatant becomes clear.

7.3 Transfer the supernatant into a new PCR tube.

Left-side selection

7.4 Add AMPure XP beads (for left-side selection; 20 µL, 8 µL, or 6 µL) to the supernatant from the right-side selection (from step 7.3), vortex mix, and incubate 5 min at room temperature.

7.5 Quick spin the tube, put on the magnet, and wait until the supernatant becomes clear.

7.6 Remove the supernatant using a P-200 pipet while the sample tube is still on the magnet.

7.7 Add 200 µL of 80% EtOH to the sample tube while the tube is still on the magnet and wait 30 sec.

7.8 Remove EtOH with a P-200 pipet while the sample tube is still on the magnet.

7.9 Repeat the EtOH washing step once more.

7.10 Quick spin the sample tube, put on the magnet, and remove the remaining EtOH completely using a P-20 or a P-10 pipet.

7.11 Air dry the beads for ~1 min with the lid kept open.

7.12 Add 30 µL of TE to the beads, vortex mix, and incubate 2 min at room temperature.

7.13 Quick spin the sample tube, put on the magnet, and collect the eluate in a new 1.5 mL microtube.

7.14 Quantify the concentration of the first-PCR product by Qubit using the dsDNA HS Kit.

7.15 Store the size-selected first-PCR product at 4°C.

8. Perform Sanger sequencing. Confirm that the sequence matches the expected sequence in the transcriptome assembly. Also, confirm that qPCR primers (inner primers) are not overlapping heterozygous polymorphic sites (e.g., SNP). Re-design qPCR primers if they overlap heterozygous polymorphic sites.

   Note: Perform Sanger sequencing from both ends of the first-PCR product by using 10 ng of the size-selected first-PCR product with the same forward and reverse primers used in

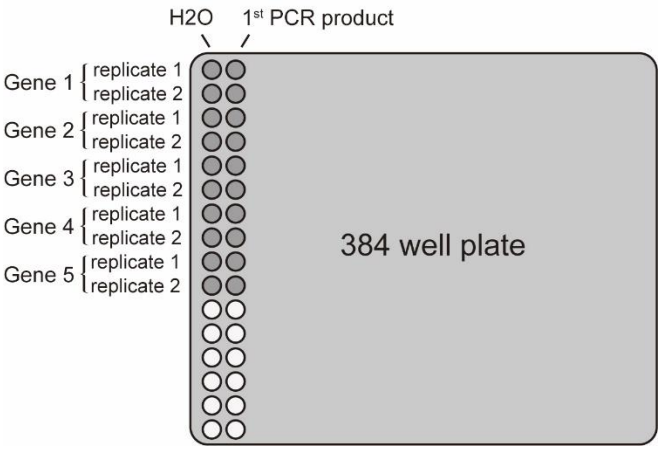
the first-PCR.

Second PCR

9. Dilute the size-selected first-PCR product five folds with H2O.
   Note: Prepare enough for the PCR cycle determination and the second PCR.
10. Prepare qPCR Master Mix for PCR cycle determination.

Cycle-determination-qPCR Master Mix	(×1 reaction)	(# of technical replication)	(# of samples: H2O and DNA)	(10% extra)		
2× QuantiTect SYBR Green	5 µL	×2	×2	×1.1	=	22 µL
Forward primer (10 uM)	0.2 µL	×2	×2	×1.1	=	0.88 µL
Reverse primer (10 uM)	0.2 µL	×2	×2	×1.1	=	0.88 µL
H2O	3.6 µL	×2	×2	×1.1	=	15.84 µL
Total	9 µL	×2	×2	×1.1	=	39.6 µL

11. Dispense 9 µL of the Cycle-determination-qPCR Master Mix in a 384 well qPCR plate as shown below and add 1 µL of the diluted first-PCR product (from step 9) or H2O.



Note: Modify the plate layout for the different number of CVG genes or the different number of technical replications.

12. Perform qPCR at [95°C for 15 min, and 30 cycles of (94°C for 15 sec, 60°C for 15 sec, 72°C for 20 sec)] with QuantStudio 7 Flex system (or an equivalent system) and determine the Ct (cycle threshold) that reaches 1/4 of the maximum fluorescence intensity.
    Note: Set the fluorescence value as Rn or raw fluorescence intensity..
13. Prepare PCR mix for the second PCR in an 8-well tube.

PCR Mater Mix (Second-PCR-mix)	(×1 reaction)
2× KAPA HiFi HotStart Ready Mix	10 µL
Forward primer (10 uM)	0.6 µL
Reverse primer (10 uM)	0.6 µL
Template DNA	1 µL
H2O	7.8 µL
Total	20 µL

14. Perform PCR with cycles and the annealing temperature determined earlier, at [98°C for 3 min, X cycles of (98°C for 20 sec, Y°C for 15 sec, 72°C for 15 sec), 72°C for 1 min, and a hold at 4°C].
15. Double size-select the second-PCR product using AMPure XP and elute in 30 µL of TE buffer.
    Note: Follow step 7, the section for "Preparation of qPCR copy number standards" for the condition of size selection using the AMPure XP beads.
16. Quantify the concentration of the size-selected second-PCR product by Qubit using the dsDNA HS Kit.
17. Analyze size distribution of the size-selected second-PCR product by Bioanalyzer using the High Sensitivity DNA Kit. Only use the size-selected second-PCR product that was confirmed to be single band for the next step (Quantitative PCR).
    Note: Dilute the size-selected second-PCR product to 0.2 ng/µL with H2O for the size distribution analysis in Bioanalyzer.
18. Store the size-selected second-PCR product at 4°C until use.
    Note: The size-selected second-PCR product will be used as the DNA copy number standard in the next step.

Quantitative PCR

1. Take 49 µL of the sheared gDNA (from step 7 of the "genomic DNA extraction" section) in a new 1.5 mL LoBind tube, add 1 µL of 50× DB stock solution and vortex mix.
2. Take DNA standards (from step 18 of the "Preparation of qPCR copy number standards" section) in a new 1.5 mL LoBind tube, adjust the concentration and volume to 1-5 ng/µL and 49 µL with H2O, add 1 µL of 50× DB stock solution and vortex mix.
3. Quantify the concentration of the sheared gDNA and the DNA copy number standards prepared in 1× DB by Qubit or QubitFlex using the dsDNA HS Kit.
    3.1 Calculate the total number of samples to make 4 independent measurements.
    Note: Each replicate consists of Qubit dsDNA HS Standard 1, Qubit dsDNA HS Standard 2 and the DNA copy number standards (e.g., for CVG transcript 1, CVG transcript 2, and CVG transcript 3).
    3.2 Make the Qubit working solution by diluting the Qubit dsDNA HS reagent 1:200 in Qubit dsDNA HS buffer.
    Note: Make enough Qubit working solution to measure the entire sample set including both the Qubit dsDNA HS Standards and DNA copy number standards.

3.3 Pre-rinse the pipet tips by aspirating and dispensing the Qubit working solution 2-3 times, then immediately dispense 190 µL (for Qubit dsDNA HS Standards) or 195 µL (for DNA copy number standards) of the Qubit working solution into 0.5 mL tubes (for Qubit) or 8-well tubes (for QubitFlex) by reverse pipetting. Close the lid to avoid evaporation of the Qubit working solution.
Note: Use a P-200 pipette.

3.4 Take 10 µL of Qubit standards or 5 µL of the sheared gDNA and the standard DNAs without pre-rinsing the pipette tip, dispense directly into the Qubit working solution and pipet up and down 2-3 times with the tip submerged below the liquid surface.
Note. Use a P-10 pipette.

3.5 Close the lid, vortex mix the tubes, spin down to collect the liquid at the bottom of the tube, and make measurement using Qubit or QubitFlex.
Note: Avoid measuring the same tube more than once because repeated measurement will cause the sample temperature to rise, resulting in the DNA concentration to display increased values.

3.6 Obtain the average concentration from the 4 independent measurements.
Note: Coefficient of variation (CV) of the 4 independent measurements should not exceed 1.5 %. If the value is over 1.5%, vortex mix the DNA samples again and repeat the measurement with Qubit.

4. Prepare DNA standard at 50,000 copy DNA/µL (STD1).

4.1 Calculate DNA copy number of each DNA standard (at 1-5 ng/µL in 1× DB) by the following formula. DNA copy number in 1 µL DNA = ((DNA concentration (ng/µL)) / (MW (g) × 10^9)) × (6.02214076 × 10^23).
Note: Molecular weight (MW) of the double-strand DNA standard is calculated by the OligoCalc program (http://biotools.nubic.northwestern.edu/OligoCalc.html).

4.2 Dilute the DNA standards 100 folds in 1× DB (results in 100-fold dilution).

4.2.1 Pre-rinse the pipet tip with 1× DB by aspirating and dispensing the 1× DB 2-3 times, then dispense 495 µL of 1× DB in a 1.5 mL micro tube by reverse pipetting.
Note: Use a P-1000 pipette.

4.2.2 Take 5 µL of the diluted DNA standards at 1-5 ng/µL (from step 2 of the "Quantitative PCR" section) without pre-rinsing the pipette tip, dispense directly into 495 µL of 1× DB in 1.5 mL micro tube, pipet up and down 2-3 times with the pipette tip submerged below the liquid surface, close the lid, vortex mix, and quickly spin down.
Note: Use a P-10 pipette.

4.3 Further dilute the DNA standards 100 folds in 1× DB (results in 10,000-fold dilution).

4.3.1 Pre-rinse the pipet tip with 1× DB by aspirating and dispensing the 1× DB 2-3 times, then dispense 495 µL of 1× DB in a 1.5 mL micro tube by reverse pipetting.

Note: Use a P-1000 pipette.

4.3.2 Take 5 µL of the 100-fold diluted DNA standards without pre-rinsing the pipette tip, dispense directly into 495 µL 1× DB in 1.5 mL micro tube, pipet up and down 2-3 times with the pipette tip submerged below the liquid surface, close the lid, vortex mix, and quickly spin down.

Note: Use a P-10 pipette.

4.4 Further dilute the DNA standard to 50,000 copy DNA/µL.

4.4.1 Pre-rinse the pipet tip with 1× DB by aspirating and dispensing the 1× DB 2-3 times, then dispense 100 µL of 1× DB in a 8-well tube by reverse pipetting.

Note: Use a P-100 pipette.

4.4.2 Calculate the amount (= X µL) of the 10,000-fold diluted DNA standard that corresponds to 5 × 10^6 copy DNA.

4.4.3 Remove X µL of 1 × DB from the 8-well tube (from step 4.4.1).

4.4.4 Take X µL of the 10,000-fold diluted DNA standard (from step 4.3.2) without pre-rinsing the pipette tip, dispense directly into 1× DB (from step 4.4.3), pipet up and down 2-3 times with the pipette tip submerged below the liquid surface, close the lid, vortex mix, and quickly spin down.

4.4.5 The prepared DNA standard at 50,000 copy DNA/µL will be STD1.

5. Prepare DNA standards STD2~STD5.

5.1 Prepare STD2 at 10,000 copy DNA/µL.

5.1.1 Pre-rinse the pipet tip with 1× DB by aspirating and dispensing the 1× DB 2-3 times, then dispense 20 µL of 1× DB in a 8-well tube by reverse pipetting.

Note: Use a P-20 pipette or a P-10 pipette (adjust to 10 µL and dispense twice for the P-10 pipette).

5.1.2 Take 5 µL of STD1 without pre-rinsing the pipette tip, dispense directly into 20 µL 1× DB in 8-well tube, pipet up and down 2-3 times with the pipette tip submerged below the liquid surface, close the lid, vortex mix, and quickly spin down.

Note: Use a P-10 pipette.

5.1.3 The prepared DNA standard solution at 10,000 copy DNA /µL will be STD2.

5.2 Prepare STD3~5 at 2,000 copy DNA/µL, 400 copy DNA /µL, and 80 copy DNA /µL

by serial dilution.

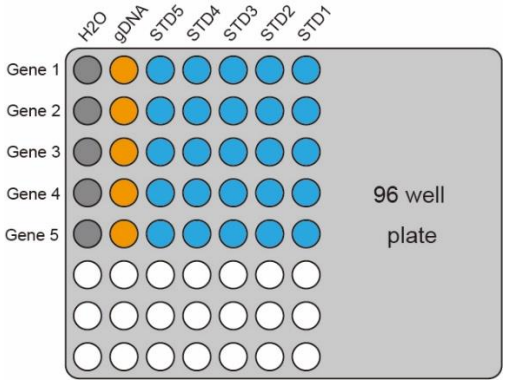
Note: Dilute STD2 to prepare STD3, dilute STD3 to prepare STD4, dilute STD4 to prepare STD5. Perform dilution as described for steps 5.1.1~5.1.3.

6. Prepare qPCR mix for genome size estimation.

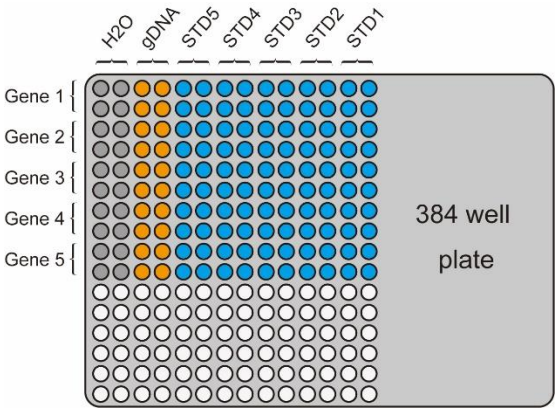
qPCR Master Mix	(×1 reaction)	(# of technical replications +0.5)	(# of samples: gDNA, DNA STDs, H2O +0.5)	
2× QuantiTect SYBR Green PCR Mix	5 µL	×4.5	×7.5	= 168.75 µL
Forward primer (10 uM)	0.2 µL	×4.5	×7.5	= 6.75 uL
Reverse primer (10 uM)	0.2 µL	×4.5	×7.5	= 6.75 uL
H2O	3.6 µL	×4.5	×7.5	= 121.5 uL
Total	9 µL	×4.5	×7.5	= 303.75 uL

7. Put a 96-well plate on a metal block on ice, dispense 9 µL × 4.5= 40.5 µL qPCR Master Mix as shown below and add 4.5 µL of H2O, gDNA (from step 1) or DNA standards (STD1~5 from steps 4 and 5) without pre-rinsing the pipette tip. Dispensing the DNA solution directly into the qPCR Master Mix and, pipet up and down 2-3 times with the pipette tip submerged below the liquid surface.

   Note: Use a P-10 pipette. Modify the plate layout for different number of CVG genes.



8. Seal the plate with an adhesive film, vortex mix the plate, and spin down the plate at 1,500 rpm for 10 sec.
9. Set the 8-channel pipet to 10 µL, pre-rinse the pipet tips by aspirating and dispensing the qPCR Master Mix 2-3 times, then dispense 10 µL into 4 replicate wells in a 384-well plate as shown below by reverse pipetting.



Note: Use a P-10 or P-20 multi-channel pipette (e.g., Picus 8-channel electronic pipette (0.2-10 µL; Sartorius). Modify the plate layout for different number of CVG genes.

10. Perform qPCR at [95°C for 15 min, 40 cycles of (94°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec)] with dissociation analysis in QuantStudio 7 Flex or equivalent system.
    Note: Confirm that the R^2 of the standard curve to be over 0.98, and the dissociation curve showing a single peak (indication of a single PCR product).
11. Obtain the concentration of the gDNA sample in DNA copy number and calculate the C-value with the following formula: Genome size (C-value) = (Amount of DNA in the qPCR reaction (ng)) / (DNA copy number from qPCR).
12. Obtain the average C-value from at least three CVG genes to determine the species' C-value. Also calculate the genome size in nucleotide base pairs by the following formula[11]: Genome size in nucleotide base pairs = (C-value) × 0.978 × 10^9

References

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