Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR

Overview

Genomic DNA (gDNA) and plasmids containing cloned target sequences are commonly used as standards in quantitative PCR. This tutorial reviews calculations that can be used for determining the mass of gDNA and plasmid templates that correspond to copy numbers of target nucleic acid sequences.

Important Notes

- It is generally not possible to use DNA as a standard for absolute quantification of RNA because there is no control for the efficiency of the reverse transcription step.
- Once prepared, it is recommended to dilute standards into small aliquots, store at –80 °C, and thaw only once before use.
- Accurate pipetting is essential because the standards must be diluted over several orders of magnitude.
- Plasmid DNA must be a single, pure species. For example, plasmid DNA prepared from *E. coli* often is contaminated with RNA, which increases the A_{260} measurement and inflates the copy number determined for the plasmid.
- Because of potential errors with pipetting and/or OD values, it is important to verify the absolute quantities of an absolute standard by some independent method. For example, one might use real-time PCR to compare several genomic DNA samples of known target quantity with a plasmid standard curve (containing the same target) to verify accuracy of the standards.
- Because plasmid (and to a lesser extent gDNA) sequences are highly abundant, they can be sources of PCR contamination. Extreme caution must be exercised when working with these DNAs to prevent their exposure to stock PCR reagents, solvents used for the dilution of PCR reagents and laboratory equipment and surfaces.

Example: Creating a gDNA Standard Curve

Prepare a standard curve in which a gene of interest is present at 300,000 copies, 30,000 copies, 3,000 copies, 300 copies and 30 copies. This example uses the human RNaseP gene, a gene that exists as a single copy per haploid genome (or 2 copies per human cell).

Step 1

Identify the genome size of the organism of interest.

The size of the human genome as determined by Celera Genomics is approximately 3 billion bp (haploid).

If the genome size of the organism of interest is not readily available, go to http://www.cbs.dtu.dk/databases/DOGS/index.html
and select the appropriate database. Below is an excerpt from the
"Abbreviated database (By name)"

Helicobacter pylori	1,667,867
Holochilus vulpinus	3,217,300,000
Homo sapiens	3,400,000,000
Hordeum vulgare	5,000,000,000
Human immunodeficiency virus type 1	9,750
Hylobates agilis	3,429,500,000

Size of human genome (haploid)

The estimate of 3,400,000,000 bp, or 3.4e9 bp (haploid), is consistent with Celera's estimate of 3,000,000,000 bp or 3.0e9 bp (haploid).

Step 2
Identify the mass of DNA per genome

Calculate the mass of the genome by inserting the genome-size value in the formula below (see page 8 for derivation of this formula).

$$m = [\,n\,]\left[\,1.096\text{e-}21\ \frac{g}{bp}\,\right]$$

where: n = genome size (bp)
m = mass
e-21 = $\times 10^{-21}$

The mass of the human genome (haploid) is calculated as follows.

$$m = [\,3.0\text{e}9\ \cancel{bp}\,]\left[\frac{1.096\text{e-}21\ g}{\cancel{bp}}\right] = 3.3\text{e}{-12}\ g$$

The calculation below converts the mass to picogram units.

$$[\,3.3\text{e-}12\ \cancel{g}\,]\left[\frac{1\text{e}12\ pg}{\cancel{g}}\right] = 3.3\ pg$$

Step 3:
Divide the mass of the genome by the copy number of the gene of interest per haploid genome.

The RNase P gene is a target that exists as a single copy gene per haploid genome (or 2 copies per human cell).

$$3.3\ pg/genome \div 1\ copy\ RNase\ P/genome = \left[\frac{3.3\ pg}{\cancel{genome}}\right]\left[\frac{\cancel{genome}}{1\ copy}\right] = \frac{3.3\ pg}{1\ copy\ RNase\ P}$$

Therefore, 3.3 pg of human gDNA contains one copy of the RNase P gene.

Step 4
Calculate the mass of gDNA containing the copy #s of interest, that is 300,000 to 30 copies.

Copy # of interest × mass of haploid genome = mass of gDNA needed

Copy #		Mass of gDNA needed (pg)
300,000	× **3.3 pg**	990,000
30,000		99,000
3,000		9,900
300		990
30		99

Step 5
Calculate the concentrations of gDNA needed to achieve the copy#s of interest. Divide the mass needed (calculated in Step 4) by the volume to be pipetted into each reaction. In this example, 5µL of gDNA solution will be pipetted into each PCR reaction. Calculate the concentration of gDNA needed to achieve the required masses of gDNA.

Copy #	Mass of gDNA needed (pg)		Final concentration (pg/µl) of gDNA
300,000	990,000	÷ **5 µL**	198,000
30,000	99,000		19,800
3,000	9,900		1,980
300	990		198
30	99		19.8

Step 6
Prepare a serial dilution of the gDNA.

For the dilutions we will use the formula,

$$C_1V_1 = C_2V_2$$

The stock concentration of human gDNA was determined by spectrophotometric analysis to be 1.2 µg/µl. Therefore, in this example, C_1 = 1.2 µg/µL or 1,200,000 pg/µL. Each dilution prepared has a final volume (V_2) of 100µL.

Dilution #1

$$\left[1{,}200{,}000 \frac{\text{pg}}{\mu\text{L}}\right]\left[V_1\right] = \left[198{,}000 \frac{\text{pg}}{\mu\text{L}}\right]\left[100\ \mu\text{L}\right]$$

V_1 = 16.5 µL

Volume of diluent = 100 µL – 16.5 µL = 83.5 µL
To achieve the final volume of 100 µL, add 16.5 µL of stock gDNA to 83.5 µL of diluent.

Note: *The diluent can be sterile 1X TE (1mM Tris, 0.1mM EDTA, pH8.0) or sterile, nuclease-free H_2O.*[1]

Dilutions 2 to 5 were calculated using the same types of calculations ($C_1V_1 = C_2V_2$) as presented above for Dilution #1.

The following table presents the calculated volumes of gDNA and diluent for all 5 dilutions.

Dilution #	Source of gDNA for dilution	Initial concentration (pg/µL) **C_1**	Volume of gDNA (µL) **V_1**	Volume of diluent (µL)	Final Volume (µL) **V_2**	Final concentration of dilution (pg/µl) **C_2**	Resulting copy # RNase P gene/ 5µl
1	stock	1,200,000	16.5	83.5	100	198,000	300,000
2	Dilution 1	198,000	10	90	100	19,800	30,000
3	Dilution 2	19,800	10	90	100	1,980	3,000
4	Dilution 3	1,980	10	90	100	198	300
5	Dilution 4	198	10	90	100	19.8	30

Example: Creating a Standard Curve with a Plasmid DNA Template[2]

Background
Prepare a standard curve in which the cloned ß-actin sequence is present at 300,000 copies, 30,000 copies, 3,000 copies, 300 copies and 30 copies. The plasmid size is 15,000 bp. The stock of plasmid DNA was determined to be 2.0 µg/µL by spectrophotometric analysis. The PCR reactions are set-up such that 5µL of plasmid DNA are pipetted into each PCR reaction.

[1] It is the users responsibility to determine whether the use of background nucleic acid will impact assay performance (ex. PCR efficiency). Background nucleic acid is DNA or RNA that can be spiked into the standard so as to mimic the biological unknown samples.
[2] It is the users responsibility to determine whether linearization of the plasmid standard with a restriction endonuclease will impact assay performance (ex. PCR efficiency).

Step 1
Calculate the mass of a single plasmid molecule.

Insert the plasmid size value into the formula below (see page 8 for derivation of this formula):

$$m = [\ n\ ]\left[\ 1.096\text{e-}21\ \frac{g}{bp}\ \right]$$

where: n = plasmid size (bp)
m = mass
e-21 = $\times 10^{-21}$

Note: *Use the size of the entire plasmid (plasmid + insert) in the calculation above instead of the size of the insert alone.*

Mass of one plasmid molecule= [15,000 bp] [1.096e-21 g/bp] = 1.64e-17 g

Step 2
Calculate the mass of plasmid containing the copy #s of interest, that is 300,000 to 30 copies.

Copy # of interest × mass of single plasmid = mass of plasmid DNA needed

For example, mass of plasmid DNA containing 300,000 copies of B-actin sequence is as follows.

[1.64e-17 g/copy] [300,000 copies] = 4.92e-12 g

The following table presents the calculated plasmid masses needed to achieve the copy numbers of interest.

Copy #		Mass of plasmid DNA (g)
300,000	× **1.64e-17 g**	4.92e-12
30,000		4.92e-13
3,000		4.92e-14
300		4.92e-15
30		4.92e-16

Step 3
Calculate the concentrations of plasmid DNA needed to achieve the copy#s of interest. Divide the mass needed (calculated in Step 2) by the volume to be pipetted into each reaction.

In this example, 5μL of plasmid DNA solution is pipetted into each PCR reaction. Calculate the concentration of gDNA needed to achieve the required masses of gDNA.

Copy #	Mass of plasmid DNA needed (g)		Final concentration of plasmid DNA (g/μL)
300,000	4.92e-12		9.84e-13
30,000	4.92e-13	**÷ 5 μL**	9.84e-14
3,000	4.92e-14		9.84e-15
300	4.92e-15		9.84e-16
30	4.92e-16		9.84e-17

Step 4
Prepare a serial dilution of the plasmid DNA.

Cloned sequences are highly concentrated in purified plasmid DNA stocks. A series of serial dilutions must be performed to achieve a working stock of plasmid DNA for quantitative PCR applications. The table on page 7 shows that the first 3 dilutions (each 1:100) were prepared so that the plasmid would be at a workable concentration, that is 2e-12 grams/μL or 1.32e5 copies/μL.

Once the plasmid is at a workable concentration, use the following formula to calculate the volume needed to prepare the 300,000 copy standard dilution (Dilution #4).

$$C_1V_1 = C_2V_2$$

Dilution #4
(*see table below for C_1, C_2, V_1 and V_2 values*)

$$\left[2\text{e-}12 \frac{\text{g}}{\mu\text{L}}\right]\left[V_1\right] = \left[9.84\text{e-}13 \frac{\text{g}}{\mu\text{L}}\right]\left[100\ \mu\text{L}\right]$$

$V_1 = 49.2\ \mu L$

Volume of diluent = 100 μL – 49.2 μL = 50.8 μL
To achieve the final volume of 100 μL, add 49.2 μL of stock gDNA to 50.8 μL of diluent.

Note: *The diluent can be sterile 1X TE (1mM Tris, 0.1mM EDTA, pH8.0) or sterile, nuclease-free H_2O.* [3]

Dilutions 5 to 8 were calculated using the same types of calculations as Dilution #4 above.

Dilution #	Source of plasmid DNA for dilution	Initial conc. (grams/μL) **C_1**	Volume of plasmid DNA (μL) **V_1**	Volume of diluent (μL)	Final Volume (μL) **V_2**	Final conc. in (g/μl) **C_2**	Resulting copy # of ß-actin sequence / 5 μl
1	stock	2e-06	10 μl	990 μl	1000 μl	2e-08	N/A
2	Dilution 1	2e-08	10 μl	990 μl	1000 μl	2e-10	N/A
3	Dilution 2	2e-10	10 μl	990 μl	1000 μl	2e-12	N/A
4	Dilution 3	2e-12	49.2 μl	50.8 μl	100 μl	9.84e-13	300,000
5	Dilution 4	9.84e-13	10 μl	90 μl	100 μl	9.84e-14	30,000
6	Dilution 5	9.84e-14	10 μl	90 μl	100 μl	9.84e-15	3,000
7	Dilution 6	9.84e-15	10 μl	90 μl	100 μl	9.84e-16	300
8	Dilution 7	9.84e-16	10 μl	90 μl	100 μl	9.84e-17	30

In the example above, dilutions 4 to 8 would be used for the quantitative PCR application.

Derivation of DNA Mass Formula

$$\mathbf{m = \left[\ n\ \right]\left[\ 1.096e\text{-}21\ \frac{g}{bp}\ \right]}$$

The formula above was derived as follows

$$m = \left[\ n\ \right]\left[\ \frac{\cancel{1\ mole}}{6.023e23\ molecules\ (bp)}\ \right]\left[\ \frac{660\ g}{\cancel{mole}}\ \right] = \left[\ n\ \right]\left[\ \frac{1.096e\text{-}21\ g}{bp}\ \right]$$

where:
n = DNA size (bp)
m = mass
Avogadros number = 6.023e23 molecules / 1 mole
Average MW of a double-stranded DNA molecule = 660 g/mole

[3] It is the users responsibility to determine whether the use of background nucleic acid will impact assay performance (ex. PCR efficiency). Background nucleic acid is DNA or RNA that can be spiked into the standard so as to mimic the biological unknown samples.

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The PCR process is covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffmann-La Roche Ltd.

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4371090 Revision A