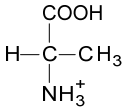
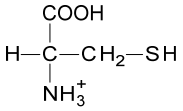
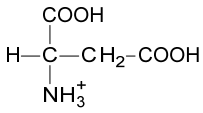
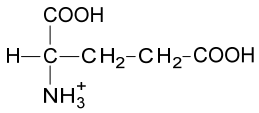
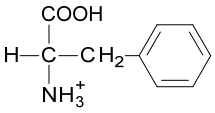
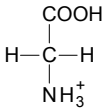
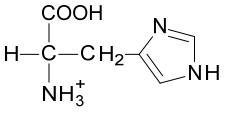
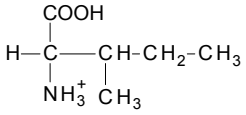
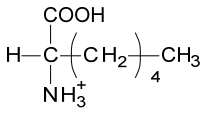
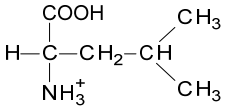
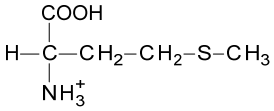
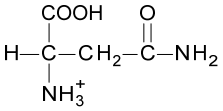
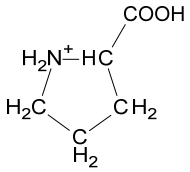
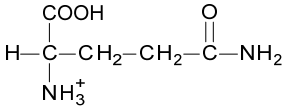
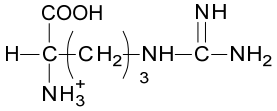
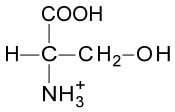
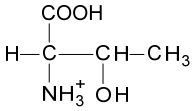
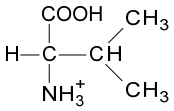
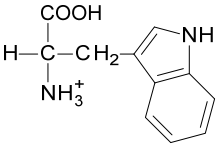
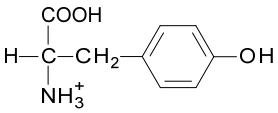
APPENDICES

APPENDIX 1. AMINOACIDS SYMBOL AND STRUCTURE

Table A1-1. Standard aminoacid symbols and chemical structures.

symbol	full name	chemical structure
A	alanine	
C	cysteine	
D	aspartic acid	
E	glutamic acid	
F	phenylalanine	
G	glycine	
H	histidine	
I	isoleucine	
K	lysine	

symbol	full name	chemical structure
L	leucine	
M	methionine	
N	asparagine	
P	proline	
Q	glutamine	
R	arginine	
S	serine	
T	threonine	
V	valine	
W	tryptophan	
Y	tyrosine	

APPENDIX 2. FLOW AND RESIDENCE TIME CALCULATION

Either for mass-flow controllers or needle valves, the flow of gases and monomer vapors into the iCVD reactor was calculated based on Equation A2-1, where F is the flow in sccm (standard centimeter cubic per minute), dP/dt is the pressure variation with time in Torr/s, T_{gas} is the temperature of the gas in the reactor (assumed to be 323.15 K) and $V_{reactor}$ is the volume of the whole system (reactor chamber and pipes), which was calculated to be 8346 cm^3.

$$F = \frac{dP}{dt} \cdot \frac{273.15}{T_{gas}} \cdot \frac{V_{reactor}}{760} \cdot 60 \qquad \textit{Equation A2-1}$$

First, the reactor is evacuated to the base pressure, the monomer or gas is entered at the set point and the butterfly valve is completely closed. For each set point, the pressure increase in the reactor over time (dP/dt) is determined (e.g., Figure A2-1 A). The slope of the tendency line corresponds to dP/dt in mTorr/s. Finally, the flow can be calculated for any other set point using the equation that is obtained from the tendency line that relates the flow with the set points (e.g., Figure A2-1 B).

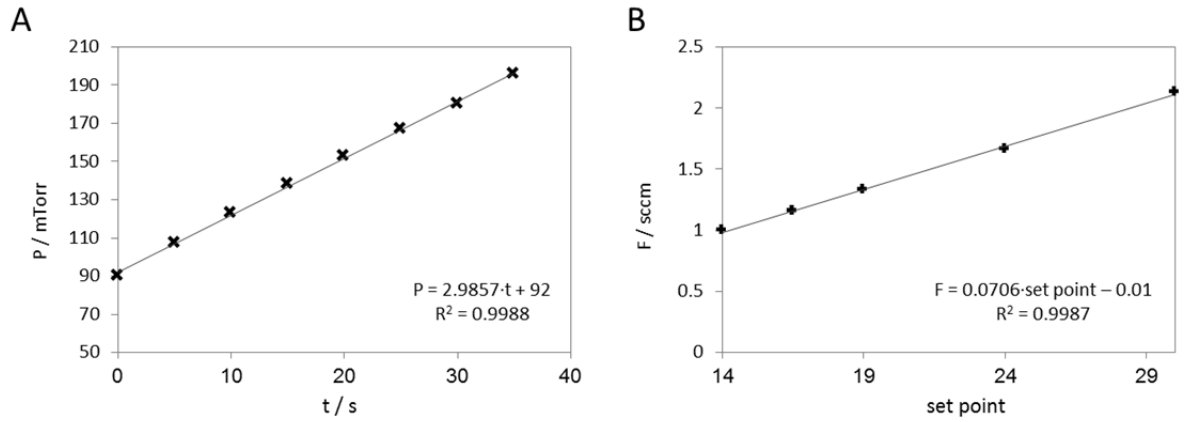


Figure A2-1. Determination of the flow rate. **(A)** Plot of pressure variation with time. **(B)** Monomer or gas flow against set point.

Residence time (τ in seconds) into the reactor is calculated with Equation A2-2, where P is the total pressure in the reactor (Torr), T_{gas} is the temperature of the gas in the reactor (assumed to be 323.15 K), $V_{chamber}$ is the volume of the reaction chamber (1493 cm^3) and F is the total flow in sccm.

$$\tau = \frac{760}{P} \cdot \frac{273.15}{T_{gas}} \cdot \frac{V_{chamber}}{F} \cdot 60 \qquad \textit{Equation A2-2}$$

APPENDIX 3. RNA ISOLATION AND QUANTIFICATION

Two different methods were used to isolate RNA from the samples. One methodology was based on sample lysis and immediate column purification, while the other added a phenol-chloroform extraction before column purification. Whereas the first one is used for RNA recovery only, the second method allows the isolation of proteins and DNA as well.

LYSIS AND COLUMN PURIFICATION

In cases where only RNA was desired (discarding DNA and proteins), lysis of the samples was performed directly with 0.5 ml of the lysis buffer provided by the peqGOLD Total RNA kit (Peqlab), which basically contains a guanidine salt. Before proceeding, lysates were homogenized by pipetting up and down 10 times with a 1 ml syringe (20 ½ G needle) or a common tip or using a pestle. Since part of the sample was lost when using the syringe, it is recommended to use the pestle and/or to pipette with a usual pipette and tip. After homogenization, samples could be stored at -80 ºC for 1 month or immediately processed. The liquid mixture was mixed with an equal volume of 70 % ethanol (nuclease-free or DEPC-treated water were used to dilute ethanol). Then, the ethanolic solution (up to 750 μl) was loaded into a HiBind RNA spin column (peqGOLD Total RNA kit, Peqlab), where RNA would bind and the flow-through liquid would be discarded after centrifugation at 10,000 x g for 1 min. Following the manufacturer's instructions, two buffers were flowed through the column successively to wash RNA. Finally, RNA was eluted with 50 μl of RNase-free water and immediately placed in ice for quantification, since RNA is very unstable.

LYSIS, PHENOL-CHLOROFORM EXTRACTION AND COLUMN PURIFICATION

Samples were lyzed with 500 μl of Trizol® (Invitrogen) or peqGOLD TriFast™ (Peqlab), two ready-to-use reagents that contain two major components: phenol and a guanidine salt. After homogenization (as indicated above), samples could be stored at -20 ºC for one month, if not used immediately. Once thawed at room temperature, 100 μl of chloroform (for each 500 μl Trizol or TriFast) were added to the lysate and the tube was vigorously shaken until an emulsion was formed. Then, the mixture was incubated for 3 min at room temperature and centrifuged at 12,000 x g for 15 min at 4 ºC. After centrifugation 3 phases appeared in the tube:

Lower phase: organic red phenol-chloroform phase, with proteins and lipids.
Interphase: with the DNA.
Top phase: colorless aqueous phase, containing the total RNA.

The upper phase was transferred into a new RNase-free tube, taking care not to aspirate DNA-interphase. Afterwards, instead of proceeding with RNA precipitation and wash (as indicated in the manufacturer's protocol), the clear supernatant was mixed with an equal volume of 70 % ethanol in

order to proceed with column purification (peqGOLD Total RNA kit, Peqlab), as explained in the previous section.

RNA QUANTIFICATION AND PURITY

The RNA amount and purity were assessed by reading in a spectrophotometer at 230 nm, 260 nm, 280 nm and 320 nm. Since RNA quantities extracted from 3D constructs were very low, systems for micro-volumes were needed. If necessary, samples were dilutes in MilliQ water. Different systems were used in this work:

- trUView™ cuvettes (Bio-Rad): these are disposable, UV-transparent cuvettes that require as little as 50 µl of sample for accurate quantitation. These cuvettes can be used in a usual spectrophotometer, as for instance Bio-Rad SmartSpec Plus spectrophotometer. The main disadvantage is that, due to the low concentration of the RNA samples, at least 25 µl of the samples are needed for good absorbance reading in this work.
- NanoDrop™ ND-1000 spectrophotometer (Thermo Scientific), NanoPhotometer™ (Implen) and TrayCell (Hellma Analytics) coupled to any spectrophotometer: these systems require a minimal sample volume for a sensitive reading (lower than 1 µl). They retain a drop of sample in a small area (Figure A3-1 A) and the lightpath length can be adjusted (Figure A3-1 B), reduced to allow smaller volumes or increased for low concentrated samples. Different technical properties of all three systems are summarized in Table A3-1.

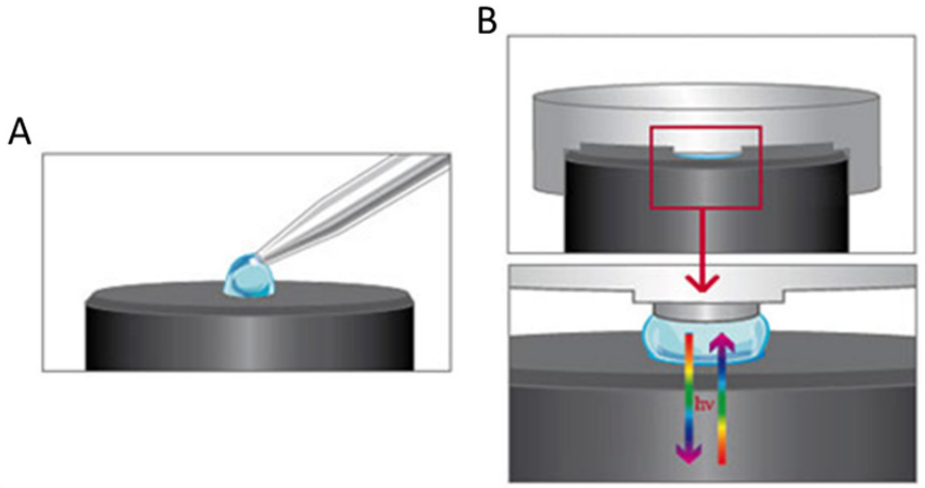


Figure A3-1. Representation of how micro-volume systems work. **(A)** A small drop is placed and retained on the surface. **(B)** A lid or arm (depending on the system) closes the sample creating a sample column with a perfectly defined length. From Implen[1].

Table A3-1. Some technical properties of low micro-volume systems.

technical features	NanoDrop™ ND-1000*[2]	NanoPhotometer™ [§1]	Hellma TrayCell [¤3]
minimal volume / μl	1	0.3	0.7
light path length / mm	1 and 0.2	0.2	1 and 0.2
virtual dilution factors	10 and 50	50	10 and 50
path length change	automatic	manual lid change	manual lid change
detection limit for dsDNA / $ng \cdot \mu l^{-1}$	2	2	2[¥]

* More recent models allow lower lightpath length (0.05 mm), and thus lower volumes (0.5 μl).
§ Optional lids can be purchased with different path length: 2, 1, 0.1 and 0.04 mm.
¤ Optional lids can be purchased with different path length: 2 and 0.1 mm. For the last one, minimum volume required is reduced to 0.5 μl.
¥ Strongly depends on the type of spectrophotometer used.

RNA concentrations were determine based on the Beer-Lambert law (Equation A3-1), which linearly correlates the absorbance (A) with the sample concentration (c) depending on the extinction coefficient of the absorber (Ɛ) and the path length (l).

$$A = \varepsilon \cdot l \cdot c \qquad \textit{Equation A3-1}$$

Therefore, RNA concentration can be calculated using Equation A3-2.

$$[RNA] = (A_{260} - A_{320}) \cdot 40 \cdot DF \cdot vDF \qquad \textit{Equation A3-2}$$

In this equation, [RNA] is the RNA concentration in μg/ml, A_{260} and A_{320} are the absorbance at 260 nm and 320 nm, respectively, DF is the dilution factor and vDF is the virtual dilution factor, in case the lightpath lengths was lower than 1 cm. RNA extinction coefficient is 0.025 $mg \cdot mL^{-1} \cdot cm^{-1}$. Therefore, an absorbance of 1 unit at 260 nm with a 1 cm pathlength corresponds to 40 μg RNA/mL, the factor that appears in the equation. Absorbance at 320 nm corrects for light scattering due to dust particles, which affects the reading at 260 nm, especially in small volumes.

RNA purity was also assessed by determination of $A_{260/280}$ and $A_{260/230}$ ratios, as indicated in Equations A3-3 and A3-4, respectively. In all cases, absorbance at 320 nm was subtracted from the readings.

$$A_{260/280} = (A_{260} - A_{320}) / (A_{260} - A_{320}) \qquad \textit{Equation A3-3}$$

$$A_{260/230} = (A_{260} - A_{320}) / (A_{230} - A_{320}) \qquad \textit{Equation A3-4}$$

For pure RNA, $A_{260/280}$ ratio is typically found between 1.7 and 2.0 for samples diluted in nuclease-free water. Lower values may indicate protein contamination, since aminoacids absorb at 280 nm. Nevertheless, this value is highly affected by pH and ionic strength. As pH increases, A_{280} decreases while A_{260} is not affected. Therefore, samples diluted in solutions buffered at pH 8 may considerably increase the range up to 2.0 - 2.2.

$A_{260/230}$ ratios should be higher than 1.8 for pure RNA, but they are often overlooked. Lower values are due to contamination by chemicals: alcohol, phenol or guanidine from lysis buffer, coming from

the extraction procedure.

In this work, RNA samples from cells grown in flasks were isolated at high concentrations (600 – 800 ng/μl) and good purity ($A_{260/280}$ ratio was around 2.0 and $A_{260/230}$ was higher than 1.8) with both methodologies (extraction plus column purification or only column purification). The procedure is problematic when dealing with constructs and hepatocyte sandwich cultures, where cell number is very low and, thus, RNA concentrations dramatically decrease (20 – 70 ng/μl). In those cases, better $A_{260/280}$ ratios were obtained when extraction step was obviated, with values typically between 1.9 and 2.0. Although the use of Trizol or TriFast seemed to give lower values, it is useful when protein isolation from the same sample is desired. Nevertheless, both procedures gave very low $A_{260/230}$ ratios for these low concentrated samples.

APPENDIX 4. QRTPCR REACTION PREPARATION AND CALCULATIONS

PREPARATION OF QRTPCR REACTION

The qRTPCR reaction is prepared by mixing a master mix solution, containing commercial SYBR Green Master Mix and primers, and the cDNA template solution for each sample, as indicated in Table A3-1.

Table A3-1. qRTPCR reaction setup for each test tube.

component	working concentrations	volume / μl
SYBR Green PCR Mater Mix*	1 x	10
primers	0.3 μM	variable (depending on stock)
cDNA template		0.75
RNase-free water		variable
total volume		20[§]

* It provides initial Mg^{2+} concentration of 2.5 mM, which was found to be optimal for all the reactions.

§ With 7500 Real-time PCR system (Applied Biosystems), 25 μl reaction volumes were used (component volumes are changed accordingly).

In particular, the different components are prepared in cold as following. Firstly, the master mix solution is formulated for each different gene. The amount of SYBR Green Master Mix is calculated in Equation A4-1 and introduced into a labeled tube with the gene name.

$$V_{SYBR} / \mu l = 10\ \mu l/tube \cdot (\#\ samples \cdot \#\ repetitions + 1) \cdot 1.1 \qquad \textit{Equation A4-1}$$

In this equation, *# samples* is the total number of biological samples; *# repetitions* is the times the qRTPCR is analyzed for each sample and gene (usually 2); one more tube is run for the blank (containing no cDNA template); 10 % extra volume is always added (factor 1.1). Depending on the number of total tubes, the 10 % extra volume can be reduced to only 5 %.

Secondly, a primer stock volume is added to the correspondent tube of SYBR solution (Equation A4-2).

$$V_{primer\ stock} / \mu l = V_{primer\ stock/tube} \cdot (\#\ samples \cdot \#\ repetitions + 1) \cdot 1.1 \qquad \textit{Equation A4-2}$$

A volume of water is calculated (Equation A4-3) and mixed with the SYBR / primer solution for each gene. Therefore, 15 μl master mix volume is dispensed into each reaction tube.

$$V_{water\ in\ SYBR\ sol} / \mu l = 15\ \mu l/tube \cdot (\#\ samples \cdot \#\ repetitions + 1) \cdot 1.1 - V_{SYBR} - V_{primer\ stock} \qquad \textit{Equation A4-3}$$

Finally, cDNA template solution is prepared for each sample, with a volume of cDNA (Equation A4-4) and a volume of water (Equation A4-5), calculated to eventually add 5 μl cDNA solution into each test tube. In these equation *# genes* is the number of genes tested (including 18s reference gene).

$$V_{cDNA} = 0.75\ \mu l/tube \cdot (\#\ genes \cdot \#\ repetitions) \cdot 1.1 \qquad \textit{Equation A4-4}$$

$$V_{water\ in\ cDNA\ sol} = 5\ \mu l/tube \cdot (\#\ genes \cdot \#\ repetitions) \cdot 1.1 - V_{cDNA} \qquad \textit{Equation A4-5}$$

When each SYBR / primer solutions and cDNA solutions are ready, test tubes are carefully filled with the corresponding 15 μl SYBR / primer solution and 5 μl cDNA solution. When Applied Biosystems cycler is used, total volume reaction corresponds to 25 μl. Therefore, 10 μl cDNA solution must be delivered into each reaction tube (Equation A4-5 has to be readjusted with 10 μl/tube, instead of 5 μl/tube).

qRTPCR CYCLER CONDITIONS

The reaction tubes are introduced into the thermocycler and subjected to the program outlined in Table A4-2.

Table A4-1. qRTPCR cycler program.

stage number	repetition	stage	time	temperature
1st	1	activation step	15 min	95 ºC
2nd	35-40	denaturation	15 s	94 ºC
		annealing	30 s	56 - 60 ºC*
		extension	30 s	72 ºC
3rd	1	dissociation		temperature ramp from 60-95 ºC

* Optimized for each primer.

Dissociation stage is performed when using SYBR Green chemistry, since SYBR Green binds all double-stranded DNA molecules, emitting a fluorescent signal on binding, which is quantified and related to the desired product. Since primer dimers and non-specific products would also produce signal, dissociation stage is required to ensure the formation of a unique product. Otherwise, the sample cannot be quantified and it is discarded. Figure A4-1 A shows the melting curves of the amplification products for 18s, bone sialoprotein and osteocalcin for different samples. All samples show defined melting curves, indicating pure products for each gene and blank samples (without cDNA template) present perfectly flat profile. In contrast, Figure A4-1 B depicts melting curves for unspecific products (different peaks or each sample).

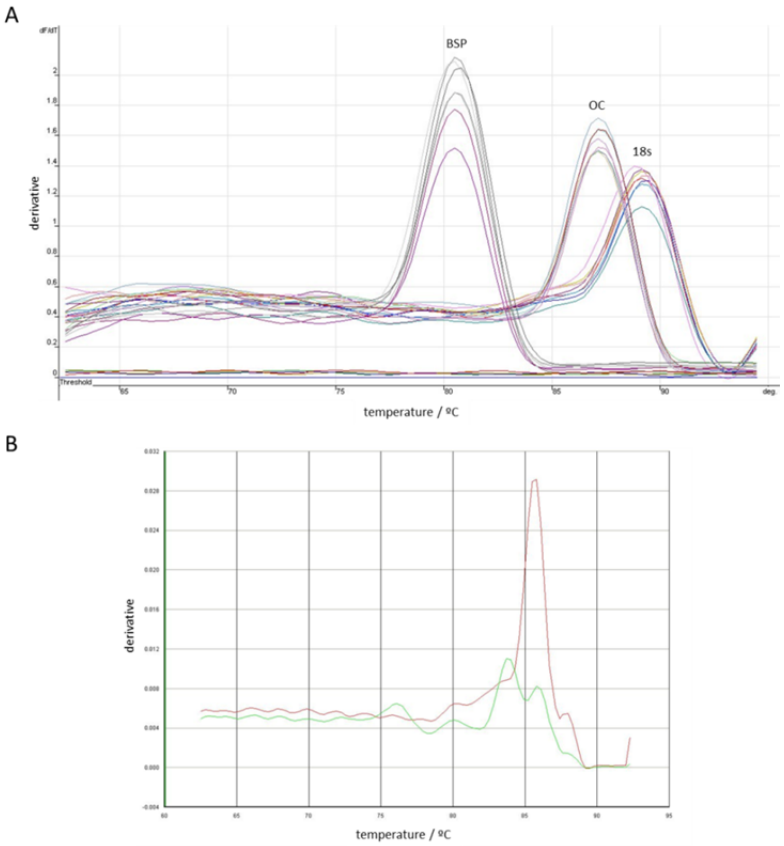


Figure A4-1. Melting curves for qRTPCR runs. **(A)** Example of melting curves of pure products for 18s, bone sialoprotein (BSP) and osteocalcin (OC). **(B)** Example of two melting curves for non-specific amplification products.

QRTPCR CALCULATIONS

From each amplification signal with single melting peak, a Ct (threshold cycle) value can be obtained. Ct is a relative measure of the concentration of target in the PCR reaction and it is the intersection between the amplification curve and a threshold line (Figure A4-2). Threshold must be set manually to ensure that the level is above the baseline, but sufficiently low to be within the exponential growth region of the amplification curve[4]. The exponential phase in Figure A4-2 A corresponds to the linear phase in Figure A4-2 B.

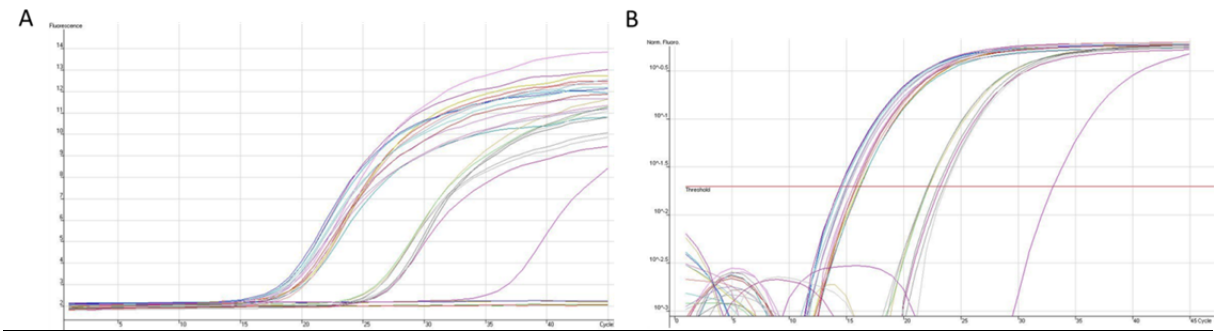


Figure A4-2. Graphical representation of qRTPCR data. Amplification plots show florescence signal against cycle number. **(A)** Fluorescence signal (baseline corrected). **(B)** Logarithm of the fluorescence signal (baseline corrected). Threshold is set at 0.02.

From the Ct values, relative quantification of the target gene is commonly calculated by using the $\Delta\Delta$Ct method. The amount of target, normalized to an endogenous reference gene (ref gene) and relative to a calibrator (cal), is given by Equation A4-6 and A4-7. The reference gene used in the results reported in this thesis is 18s, which was already proved in other works to be invariant across different 2D and 3D cultures in collagen and self-assembling peptide hydrogels[5].

$$\text{Amount of target} = 2^{-\Delta\Delta Ct} \qquad \textit{Equation A4-6}$$

$$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{cal} = (Ct_{sample,\ target\ gene} - Ct_{sample,\ ref\ gene}) - (Ct_{cal,\ target} - Ct_{cal,\ ref\ gene}) \qquad \textit{Equation A4-7}$$

Nonetheless, the $\Delta\Delta$Ct method assumes that the amplification efficiencies of the target and reference must be equal and 100%. Otherwise, the method must not be used. Conversely, a more general calculation must be applied[6]. Normalized expressions (NE) for the sample and calibrator must be calculated as indicated in Equation A4-8[7] and both NE must be divided to obtain the amount of target related to calibrator (Equation A4-9).

$$\text{Normalized expression} = \frac{E_{ref\ gene}^{\ Ct_{ref\ gene}}}{E_{target\ gene}^{\ Ct_{target\ gene}}} \qquad \textit{Equation A4-8}$$

$$\text{Amount of target} = \frac{NE_{sample}}{NE_{calibrator}} \qquad \textit{Equation A4-9}$$

In order to use these expressions, amplification efficiency (E) for each gene must be determined. PCR amplification efficiency is the rate at which a PCR product is generated. The slope of a standard curve is commonly used to estimate the PCR amplification efficiency of a real-time PCR reaction, using Equation A4-10. A real-time PCR standard curve is graphically represented as a semi-log regression line plot of Ct value against the logarithm of cDNA amount[8] (Figure A4-3). A standard curve slope of –3.32 indicates a PCR reaction with 100% efficiency that corresponds to an efficiency value (E) of 2 (as it is defined in the expressions here developed).

$$E = 10^{-1/slope} \qquad \textit{Equation A4-10}$$

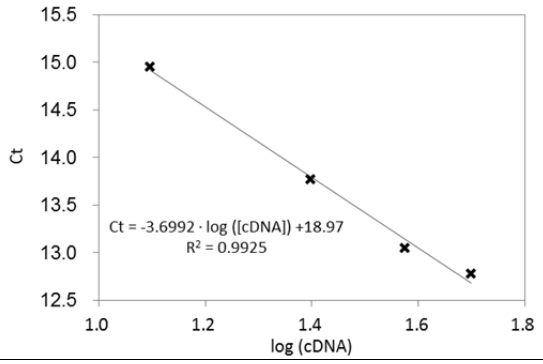


Figure A4-3. Standard curve for 18s gene. Efficiency can be calculated from the slope of the line.

APPENDIX REFERENCES

1. Implen. *NanoPhotometer® P-Class: How it works.* March 2012 - last update (http://implen.com/nanophotometer/how-it-works.php).

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