PCR and quantitative-PCR protocol

Description:

qPCR is a method of quantifying DNA based on PCR (polymerase chain reaction). qPCR tracks target concentration as a function of PCR cycle number in order to derive a quantitative estimate of the initial template concentration in a sample. In the case of conventional PCR, it uses a polymerase, dNTPs (what it is), and two primers designed to match sequences within a template.

Quantification workflow (standard curve in cartoon & unknowns)

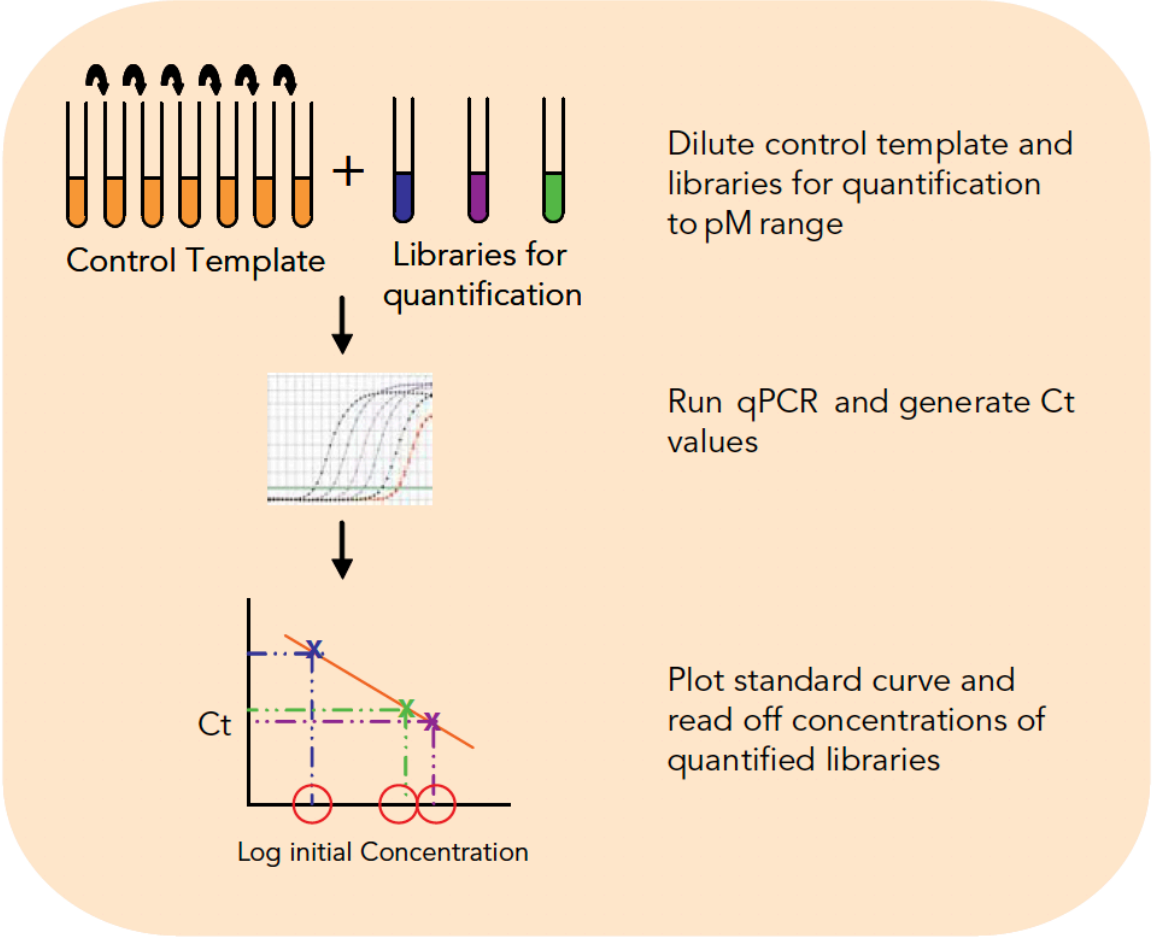


Figure 1 qPCR Quantification Workflow

Important notes before starting:

1. During qPCR setup, it is important to avoid DNA cross-contamination. Clean the set-up area, including all equipment to be used, thoroughly with (10% bleach). Recommended using a dedicated set of pipettes for qPCR to minimize contamination. Also avoid dnases so change gloves frequently.

2. The accuracy of qPCR is highly dependent on accurate pipetting and thorough mixing of solutions. Take extra care to avoid pipetting errors during qPCR set up and when preparing templates for clustering.

Materials

0.1N NaOH
10% bleach
70% ethanol
0.2 – 0.5 mL Microcentrifuge tubes
Control template 10nM (product info)
Benchtop centrifuge with swing out rotor
Benchtop microcentrifuge
qPCR machine (QIAGEN machine)
vortex
qPCR primer 1
qPCR primer 2
pipettes
SYBR qPCR master mix universal- Thermo Scientific k0222 Quartzy
Total RNA: 0,01 - 5 μg (max 12,5 μl, 2 μg standard) - sample
Oligo dT – Sigma Aldrich O4387 Quartzy
dNTP (2mM) – Fischer Scientific r0192 Quartzy
mQ
5X reaction buffer - Sigma Aldrich p9367 Quartzy
RNasin 40 U/μl - Promega N2615 Quartzy
RevertAid enzyme- Fischer Scientific Quartzy
Dnase free water – Fischer Scientific BP561-1 Quartzy

Equipment

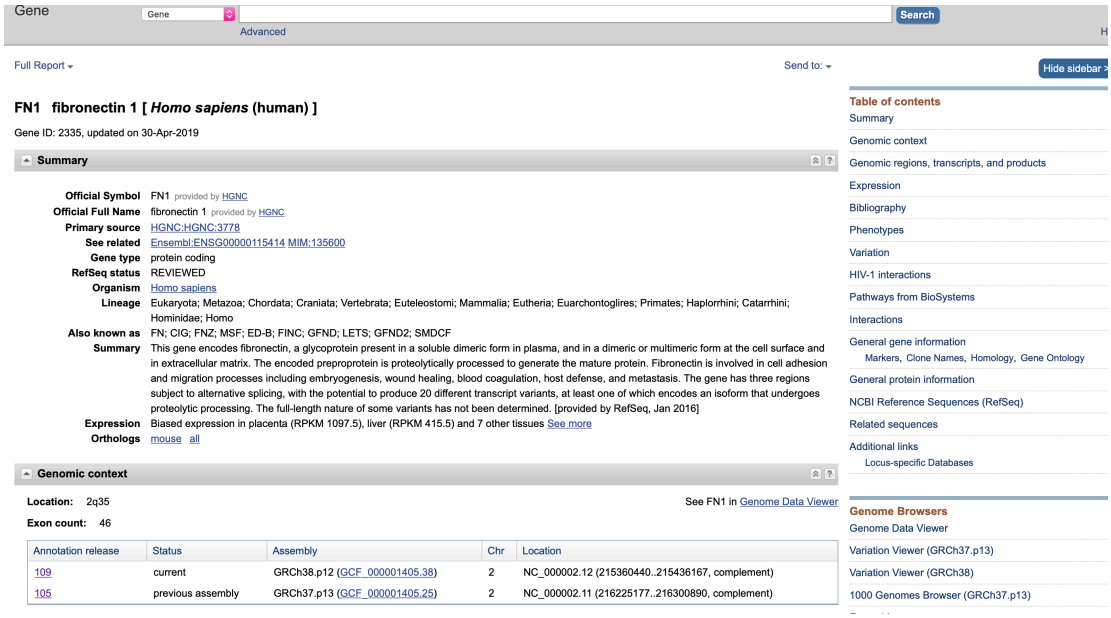
DNA Engine Thermal Cycler (conventional PCR) QIAGEN for QPCR (peytonlab owned with Forbes)

Protocol to design primers

1. Go to literature and see if someone has run PCR in it.
2. Only if not follow steps below.

Steps for primer design

1. Go to Pubmed gene database and search for your gene of interest. Filter by species in the right-hand corner of the screen. Click on your gene of interest and scroll down until you find the NCBI reference sequence (RefSeq) for your gene (e.g. NM_2034483). Click there and in the next screen you will see a link to “pick primers” in the right corner of the screen.



2. Figure. Pubmed for FN1 human gene

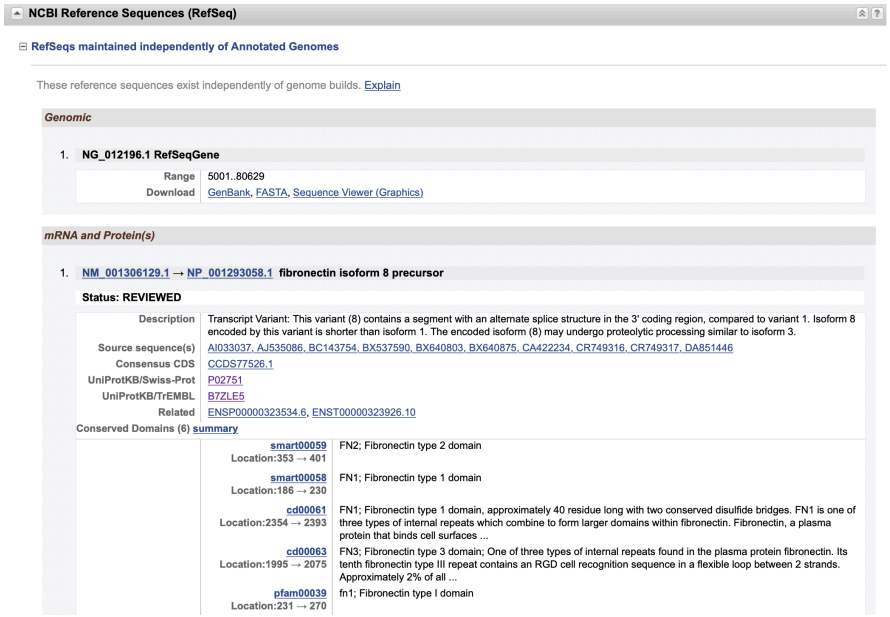


Figure. NCBI ref seq page

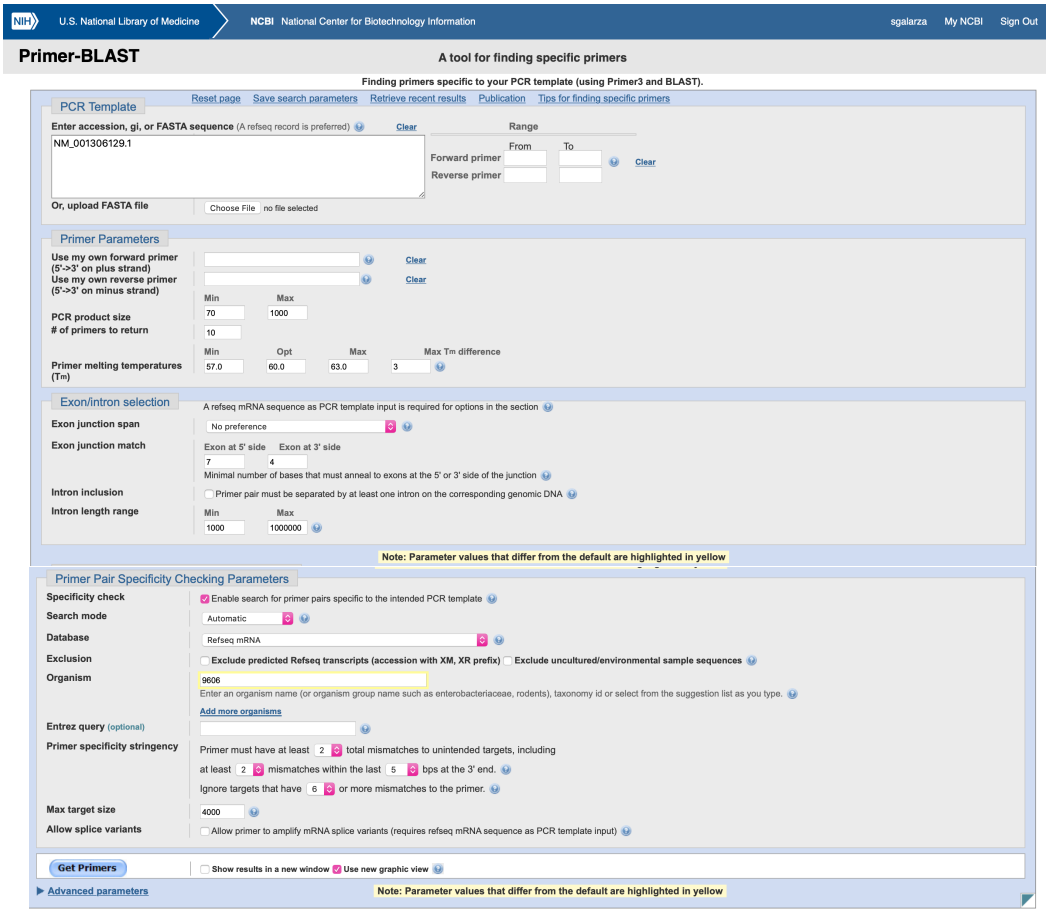


Figure. Pick primers page

3. Select qPCR primer parameters

   i. PCR product/amplicon size – for efficient amplification, design the primers so that the amplicon is between 70 and 200 bp long
   ii. Number of primers to return – this is up to you, depending on how many options you want to choose from
   iii. Melting temperature – as a rule of thumb, aim for a minimum of 60 C and a maximum of 63 C. The ideal melting temperature is 60C with a maximum difference of 3C in the melting temperatures, Tm, of the two primers.
   iv. Exon/Intron selection – to avoid amplification of contaminating genomic DNA, design primers so that one half of the primer hybridizes to the 3' end of one exon, and the other half to the 5' end of the adjacent exon. To do this, simply select "Primer must span an exon-exon junction". You do not need to change the other settings.
   v. Primer pair specificity checking parameters – use the default settings. The program will use the RefSeq mRNA sequence from the organism you selected to design the primers.
4. Checking the output screen
   i. Make sure the 3' end of the primer contains a C or G residue, because T and A residues bind more easily to DNA in a non-specific way
   ii. Aim for a GC content of around 40-60% to ensure maximum product stability
   iii. Avoid self-complementarity to decrease the possibility of primer-dimer formation. Ideally, the primer should have a near random mix of nucleotides.
   iv. Pick the best two or three primers and test them. Good luck!

Table 1. General recommendations on designing PCR primers.

Dos	Don'ts
• 15–30 nt long • T_m 55–70°C (within 5°C, for two primers) • 40–60% GC (with uniform distribution) • One C or G at 3′ end	• Secondary structure (complementarities) • Direct repeats • More than three G or C at 3′ end

Making cDNA - RT-PCR using RevertAid Premium reverse transcriptase (Fermentas)

Note: **Always work on ice !!! and with gloves and tips with filter!** *If DNA contamination can be a problem, add a negative control without RevertAid Premium enzyme as a negative control (DNA background)*

Mix together: Total RNA: 0,01 - 5 µg (max 12.5 µl, 2 µg standard)
Oligo dT 1 µl
dNTP (2mM) 1 µl
mQ X µl
Total volume: 14.5 µl

Incubate @ 65°C, 5 min
Put on ice for 2 min and spin down (short spin)
Add to previous: 5X reaction buffer: 4 µl
RNasin 40 U/µl: 0.5 µl
RevertAid enzyme: 1 µl

Incubate @ 50°C, 30 min
Incubate @ 85°C for 5 min to inactivate enzyme
Dilute 1/5 (add 80 µl mQ) and use 2 µl of this dilution for qPCR
Now that you are done with making cDNA you are ready to make samples to run PCR (add make cDNA ASAP, how long can we stored the cDNA for in the freezer?)

Procedure:

I. Prepare reaction mix

1. It is important to make a master mix to minimize pipetting errors.
2. Prepare the SYBR master mix reaction mix as follows:

Consumable	µl/well	µl/plate (master mix for 110)
KAPA SYBR FAST Master Mix Universal (2x)	10	1100
qPCR Primer 1.1 (10 µM)	0.2	22
qPCR Primer 2.1 (10 µM)	0.2	22
Water	7.6	836
	18	1980

3. Mix gently but thoroughly
4. Place the reaction mix on ice and protect it from light until use.

II. Prepare samples

Example of: QPCR with 15 µl reaction volume

Note: To minimize pipetting, always work with a primer pair mix. Optimal primer concentrations can vary, but as a standard it is 1 µl of each primer stock (100µM) in 100µl dnase free water. This results in a final concentration of 300nM in the reaction.

1x reaction
7.5 μl SYBR mix
1 – x μl template (for reactions use normally 1μl, but up to 3 μl can be used w/o observing an inhibitory effect)
4.5 μl primerpair mix
Dnase free H_2O up to 15 μl (total volume)

Run QPCR as usual (**tell the machine that the reaction size is 15μl!**: follow steps in running a protocol)

Preparing samples for qPCR if starting from DNA:

37.5 microliters DNASE, RNASE free water
10 microliters HF phusion Buffer
0.5 microliters forward primer (10 picomoles) (50 micromolar stock concentration)
0.5 microliters reverse primer (10 picomoles) (50 micromolar stock Concentration)
10-20 ng plasmid cDNA template, 500-1000 ng genomic cDNA template (~.1 microliters usually)
1 microliter DNTPs (1 micromolar working concentration)
0.5 microliters of phusion polymerase

1.) Add above ingredients in same order as written to pcr tube. If you are doing multiple pcr reactions, multiply all components by X number of reactions (e.g. 5 reactions, 10 reactions) and make a “master mix” for pcr reaction and aliquot 50 microliters into each pcr tube
2.) PCR program on thermocycler
   a. 1) 95 C for 1 minute
   b. 2) 95 C for 30 seconds
   c. 3) 5 degrees below primer melting temperature for 45 seconds
   d. 4) 72 C for 30 seconds/1000 BP of PCR product
   e. 5) Go to step 2 35 times
   f. 6) 72 C for 10 minutes
   g. 7) 10 C forever

Running a protocol QPCR in QIAGEN
Note: The incubation times and temperatures for the different steps of the PCR reaction depend strongly on the length and composition of the primers, the length of the DNA fragment you would like to amplify, the type of DNA polymerase used for the amplification, and the PCR instrument

1. Double-click the Rotor-gene Q series software icon on the desktop. Select “Advanced” tab in the “New run” dialog box that appears
2. To create a new template, select “Empty Run” and then click “New” to enter the “New Run Wizard”
3. Select 72-well rotor as the rotor type. Confirm that the locking ring is attached, and check the “Locking Ring attached” box. Then click “Next”

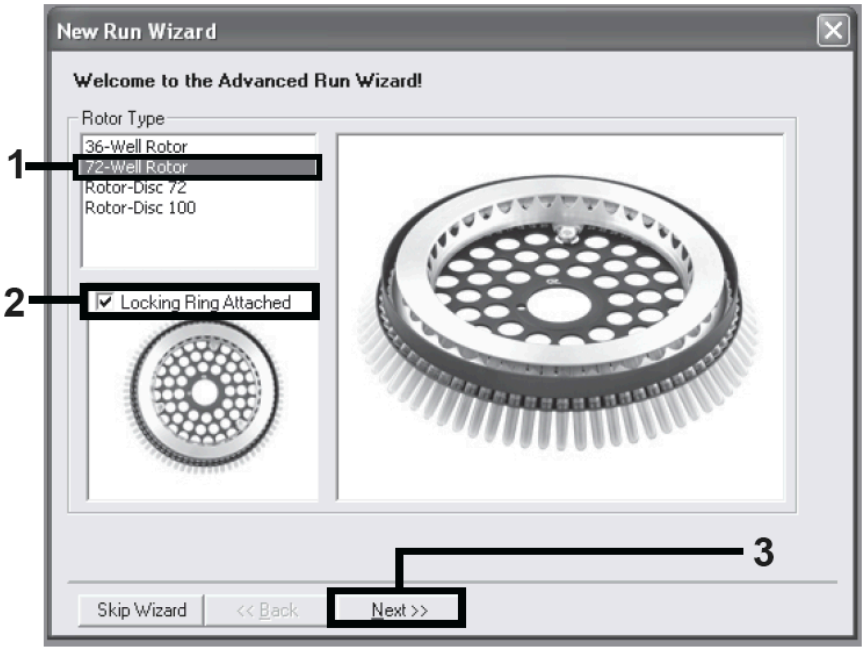


Figure 1. The “New Run Wizard” dialog box.

4. Enter the name of the operator. Add notes and enter the reaction volume (usually 15 microliters). Ensure that Sample layout reads 1, 2, 3,… and “Apply ambient air correction” is selected

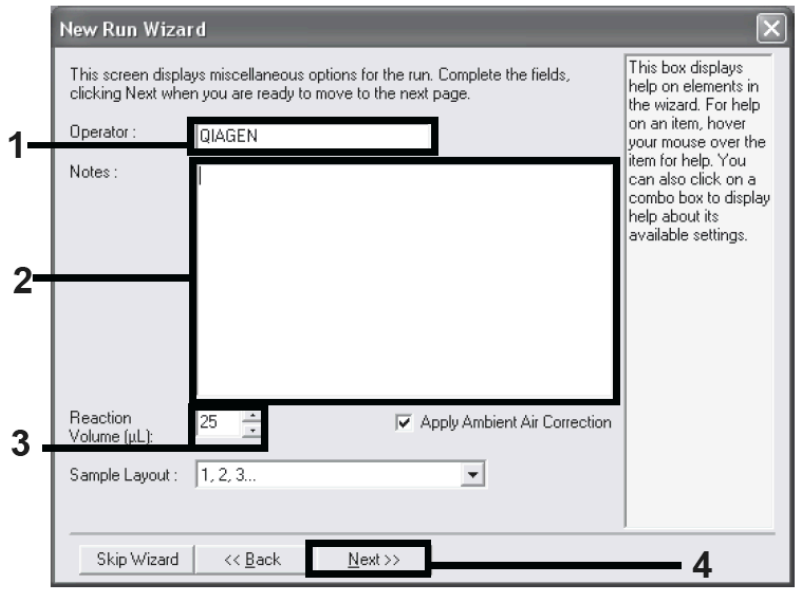


Figure 2. Setting the general assay parameters.

5. Click the “Edit profile” button in the next “New Run wizard” dialog box and program the temperature profile according to the information in the following steps

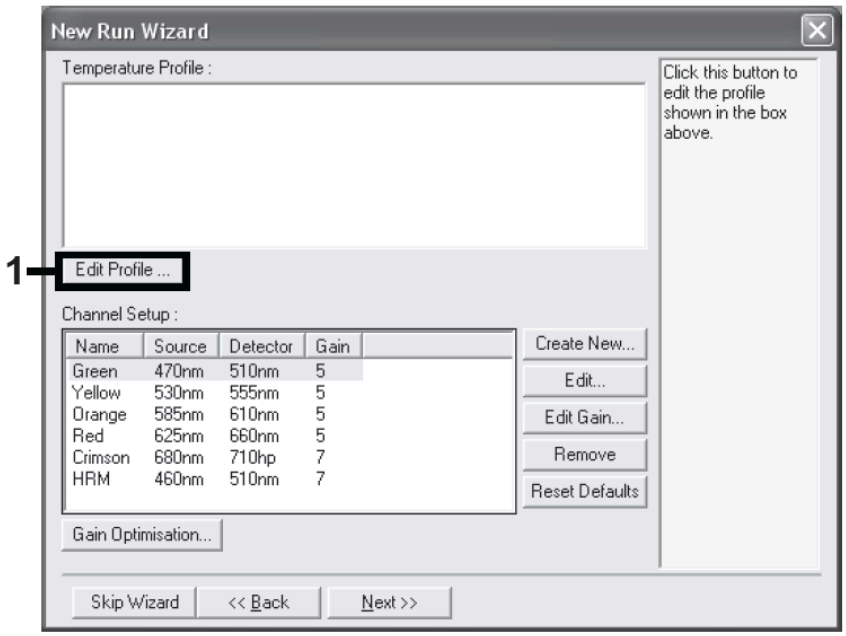


Figure 3. Editing the profile.

6. Click the “Insert after” button and select “New Hold at Temperature”

Figure 4. Initial incubation step at 95°C.

7. Change the Hold temperature to 95C, and the Hold time to 10 mins. Click the “Insert After” button and then select New cycling

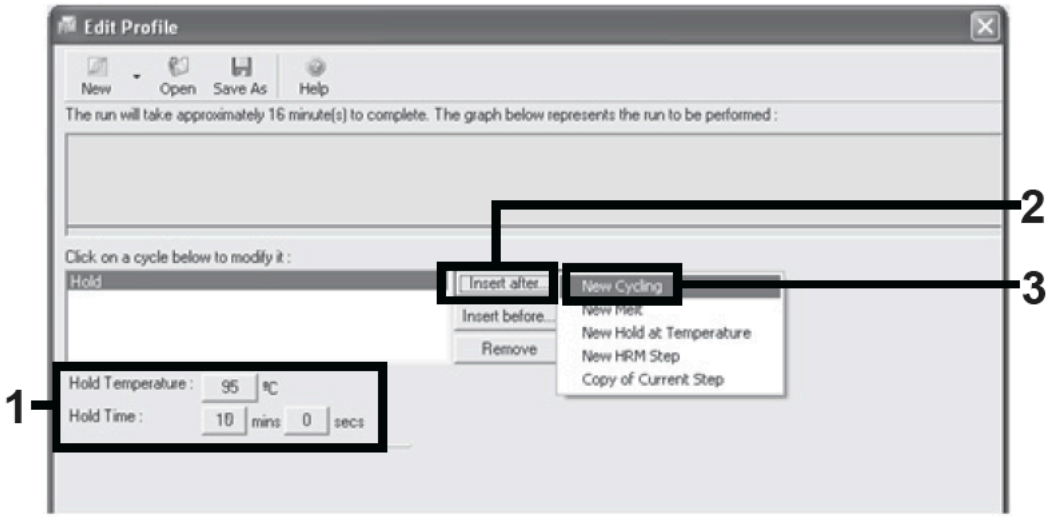


Figure 5. Initial incubation step at 95°C.

8. Change the number of cycle repeats to 40. Select the first step, and set to 95C for 30 seconds

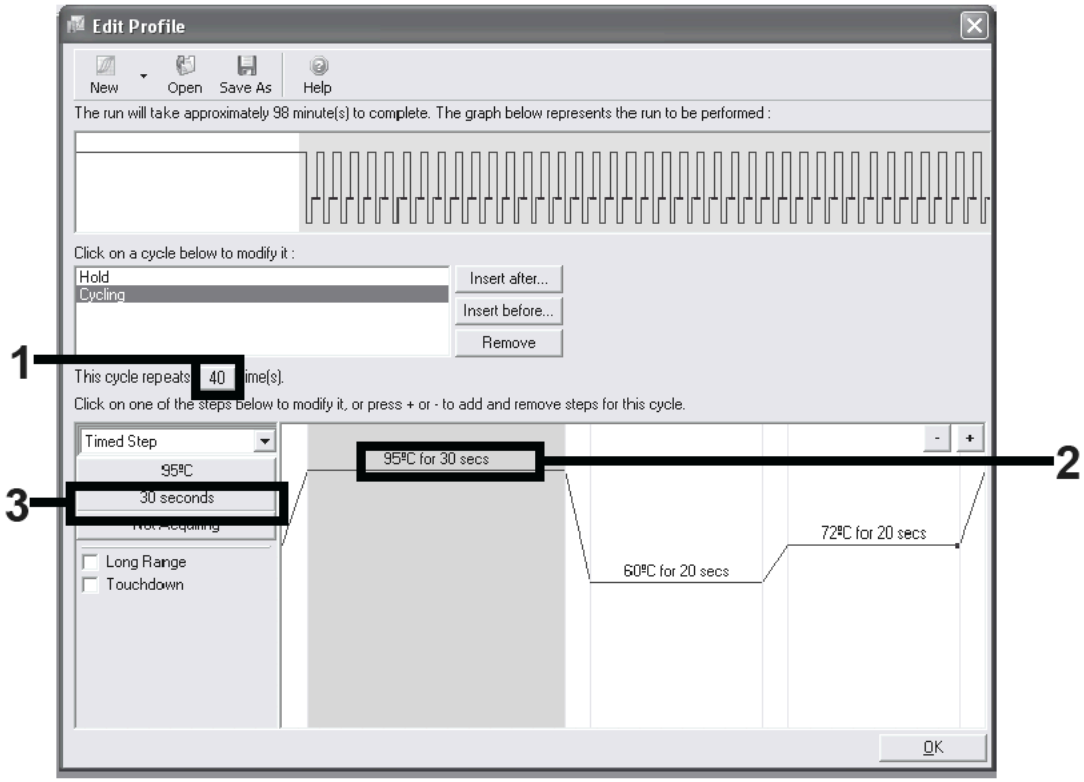


Figure 6. Cycling step at 95°C.

9. Highlight the second step, and set to 61C for 60 seconds. Enable data acquisition during this step by selecting the “Not acquiring” button

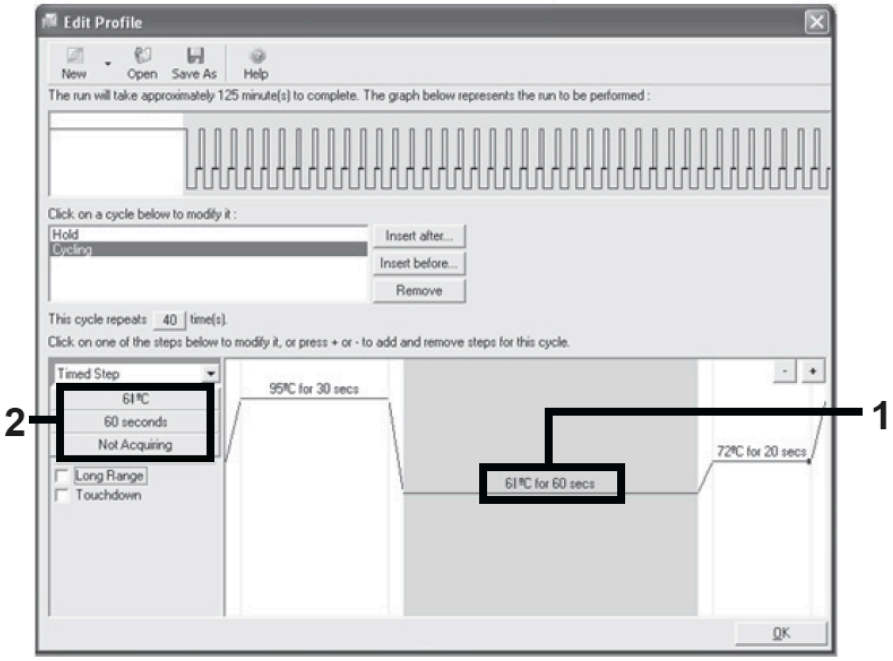


Figure 7. Cycling step at 61°C.

10. Set green and yellow as acquiring channels by selecting the “> “ button to transfer these from the available channels list and click OK

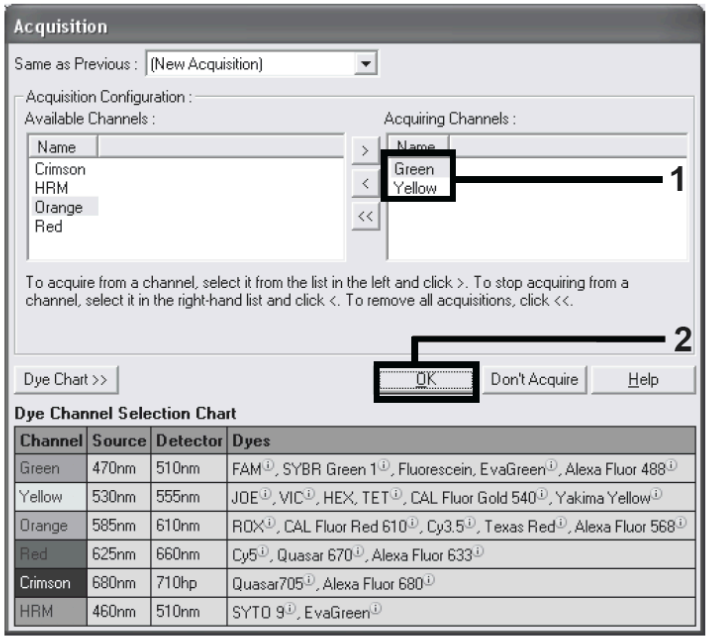


Figure 8. Acquiring at cycling step of 60°C.

11. Highlight the third step and delete by cliking the "-" button. Click OK

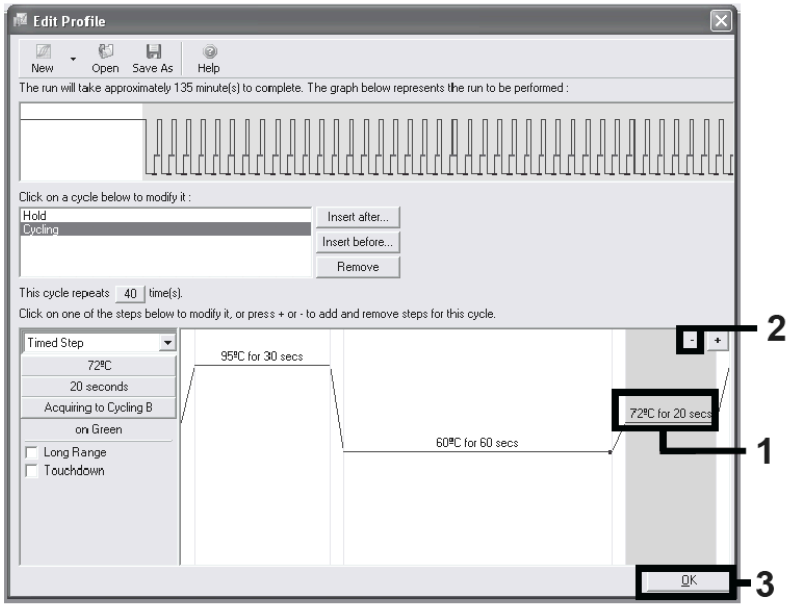


Figure 9. Removal of extension step.

12. In the next dialog box, click the "Gain optimization" button

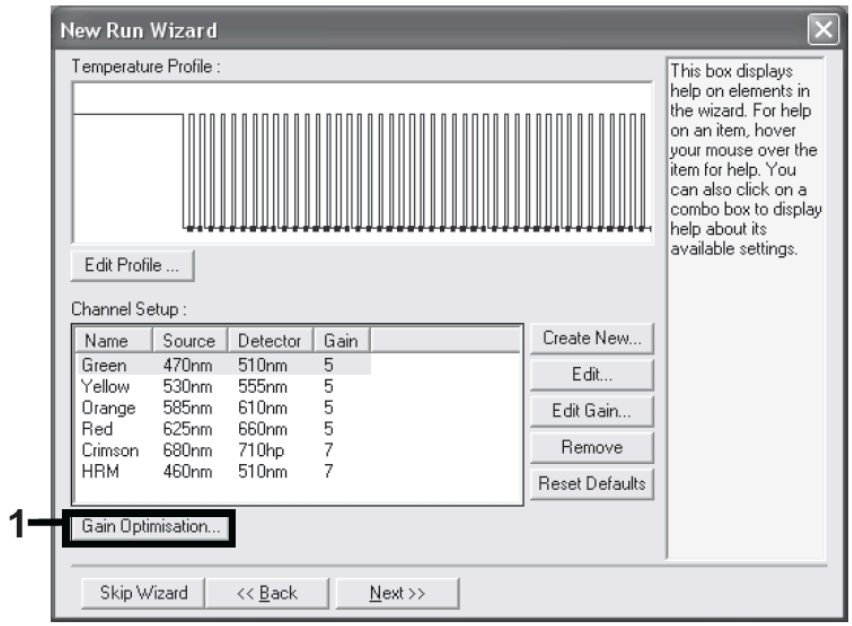


Figure 10. Gain optimization.

13. Click the "Optimise acquiring" button. Channel settings are then displayed for each channel. Accept these default values by clicking OK for both channels

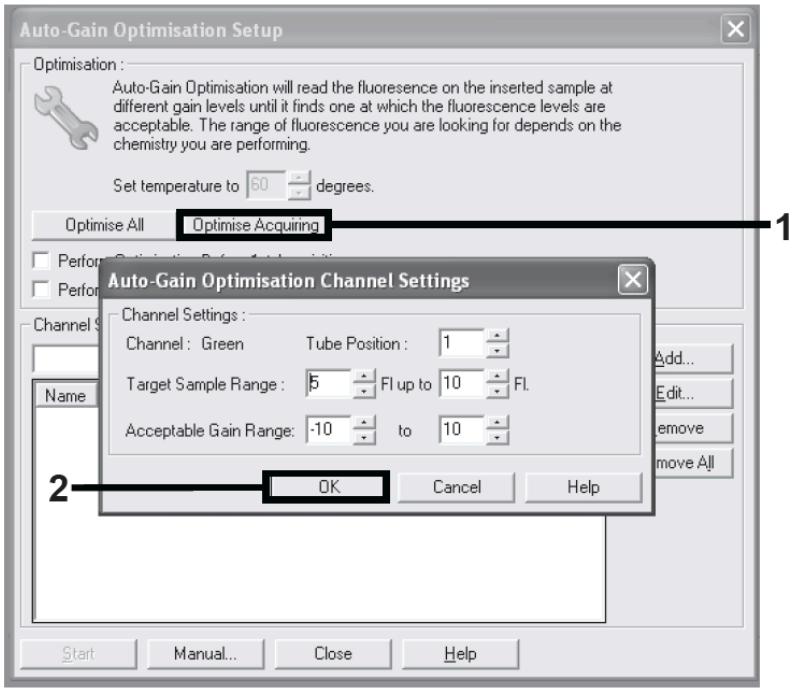


Figure 11. Auto-gain optimization for the green channel.

14. Check the “Perform optimization before 1st acquisition “ box, then click the “Close” button to return to the wizard

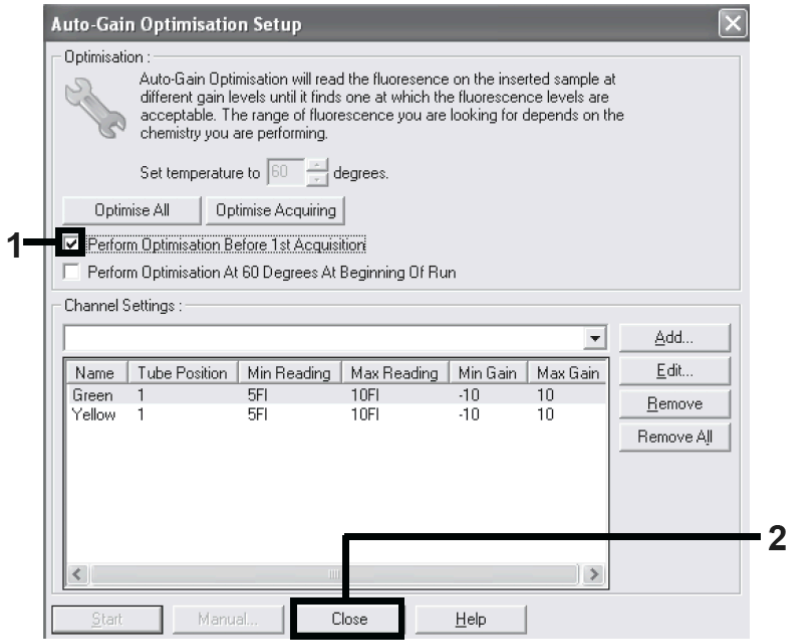


Figure 12. Selection of green and yellow channels.

15. Click “next” to save the template in an appropriate location by selecting “save template”

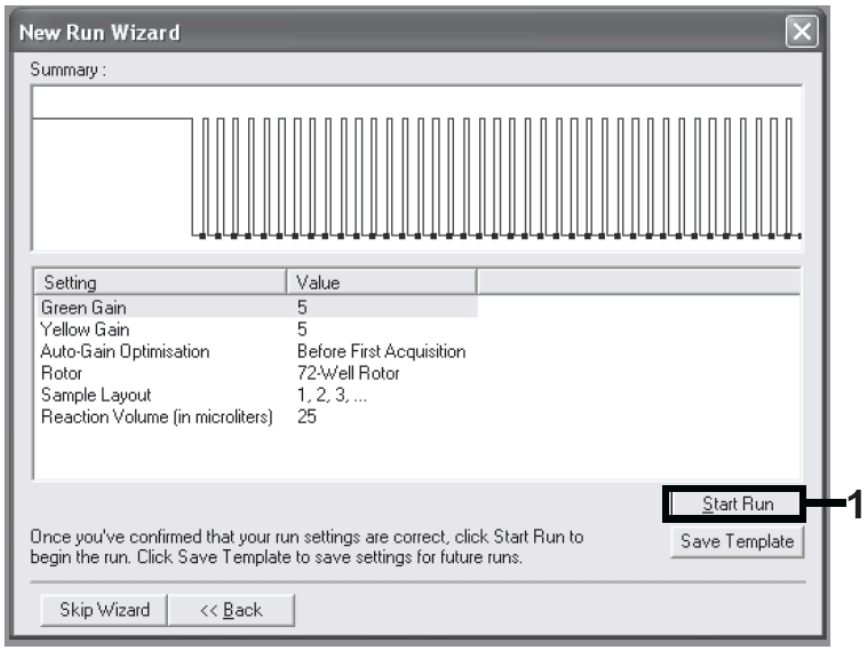
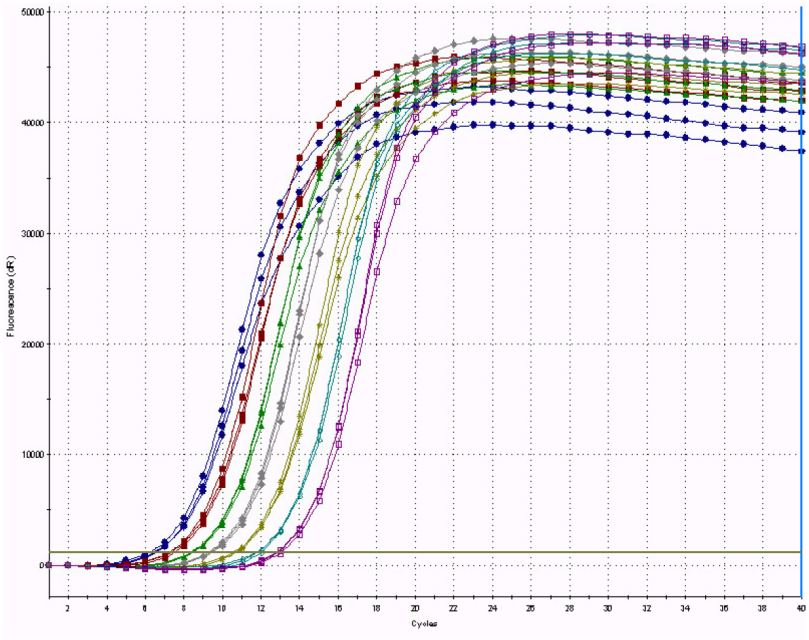


Figure 13. Starting the run.

16. Check the summary and click “Start Run” to save the run file and start the run
17. After the run starts a new window appears in which you can either enter sample names now or click “Finish” and enter them later by selecting the “Sample” button during the run, or once the run is complete.

III. Analyze

1. The final step in the qPCR procedure is to analyze the quantified samples
2. Check the NTC tubes for any amplification. There should be no amplification, but data is acceptable if any amplification is >10 cycles after your last control template amplification.
3. Ensure that there is good amplification for the control template (equipment protocol recommended to remove any bad replicates)



4. *ıre 3 Example of Control Template Amplification*
5. Generate a standard curve from the control template by plotting the Ct values against the log initial concentration-

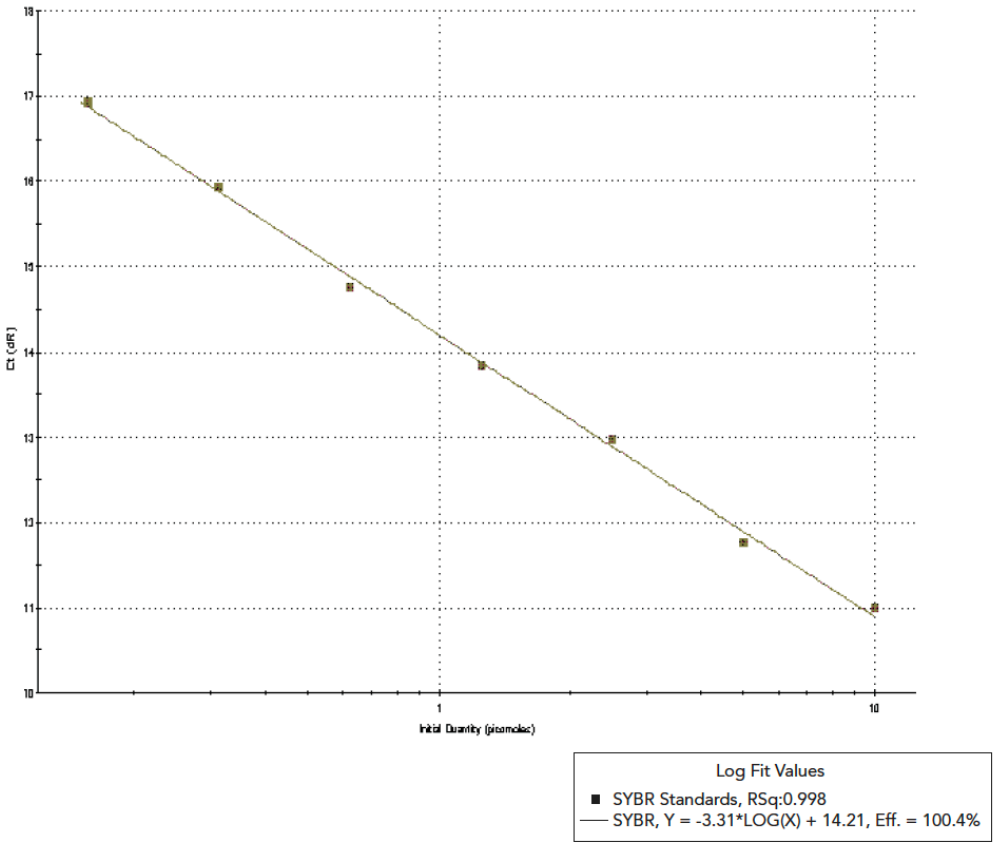
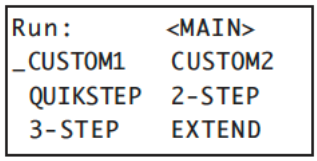


Figure 4 Example of Standard Curve

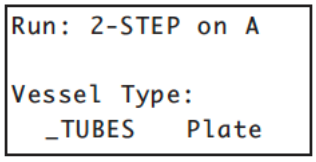
6. Ensure that the efficiency of amplification of the control template is 90–110% (a slope of -3.58 to -3.10) and that the R2 >0.9. If not, reassess the datapoints you are using to calculate the standard curve.
7. Lock the threshold fluorescence based on the standard curve.
8. Ensure that there is good amplification for the unknown samples (equipment protocol recommends removing any bad replicates)
9. Calculate the initial concentration of your unknown samples based on the standard curve generated from the control template dilutions.

I. Running a protocol PCR DNA thermocycler (conventional PCR)

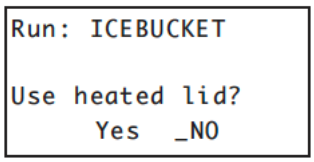
1. Running a protocol on the DNA Engine cycler involves three steps:
    i. Choosing a stored protocol to run
    ii. Choosing a block to run it on (if the dual alpha unit is installed)
    iii. Setting up the temperature control method
2. With the Main Menu displayed, select *Run,* then press <Proceed>.
3. Use the <Select> keys to scroll through the listed protocols



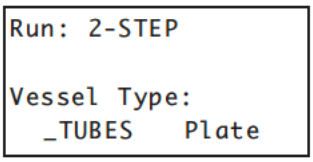
4. If a dual alpha unit is installed one of the blocks must be designated to run the protocol
5. The first available block is automatically designated when a protocol is chosen
6. Press <Block> to choose a different block
7. The selected block's letter is identified in the upper right-hand corner of the screen



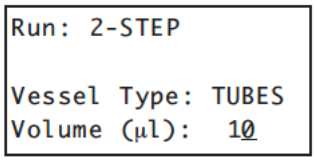
8.
9. When the protocol is selected, one or more screens will be displayed asking for information needed to set up the block's temperature control method
10. The DNA Engine cycler can control the block's temperature in two ways: block control or calculated control
11. Setting up a block control protocol: a single screen asking about use of the heated lid will be displayed



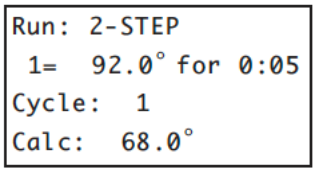
12.
13. Select *Yes* or *No,* then press <Proceed>. The protocol will begin running
14. Setting up a calculated-control protocol: three screens will be presented
    i. A screen asking for sample vessel information



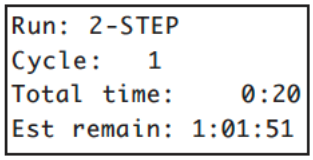
    ii. Select from the options, then press <Proceed>.
    iii. A screen asking for the sample reaction volume



    iv.
    v. Use keypad to enter sample volume in microliters, then press <proceed>. If sample vessels are sealed with oil or wax, include the volume of the oil or wax in the total sample volume entered. *Note:* specify 10uL for any volume less than 10 uL
    vi. A screen asking about the heated lid. Select *Yes* or *No* , then press <Proceed>. The protocol will begin running
15. During a protocol run, a runtime screen will be displayed



16. The screen lists the protocol name (2-STEP in the example above), protocol step that is running (1), cycle number (1), method of temperature control (Calc), and the block temperature for block-control protocols (68.0). When the step's target temperature is reached, a timer begins running in the lower right-hand corner of the screen. The first digit is the minutes elapsed; the two digits after the colon are the seconds elapsed.
17. Press the right <Select> key to see a screen listing the cycle number, time elapsed so far for the protocol run, and estimated remaining time left in the run



18. This screen is also displayed only as long as the key is pressed. The runtime screen returns when you stop pressing the key.
19. When the protocol run finishes, a long beep sounds, and a notification screen is displayed

```
Run: 2-STEP
  PROGRAM COMPLETE

Total time:   50:31
```

20.
21. Certain error messages may also be displayed in this screen. Press <Proceed> to return to the main menu

Appendix.

Specifications of the DNA engine thermal cycler

Specifications

Thermal range:	–5° to 105°C, but no more than 30°C below ambient temperature (4° to 100°C for the Slide Chambers Alpha unit)
Thermal accuracy:	±0.3°C of programmed target at 90°C, NIST-traceable
Thermal uniformity:	±0.4°C well-to-well within 30 seconds of arrival at 90°C (for most Alpha units; see specifications for individual Alpha units)
Ramping speed:	Up to 3°C/sec for all single- and dual-block Alpha units; up to 1.2°C/sec for the Slide Chambers Alpha unit.
Sample capacity:	Varies with installed Alpha unit
Line voltage:	DNA Engine® cycler: 100–240 VAC rms (no adjustment needed among voltages within these ranges)
Frequency:	50–60 Hz single phase
Power:	DNA Engine cycler: 850 W maximum
Fuses:	Two 6.3 A, 250 V, 5 x 20 mm
Displays:	One 20 x 4 LCD alphanumeric display
Ports:	One 25-pin 8-bit parallel interface printer port One 9-pin RS-232 serial port for printer or remote use One IEEE-488 bidirectional general purpose interface bus
Memory:	400 typical programs in up to 12 individual folders

Control Panel

(Figure 2-2)