

DART

DART-PCR Version 1.0

Introduction

DART-PCR provides a simple means of analysing real-time PCR data from raw fluorescence data. This allows an automated calculation of amplification kinetics, as well as performing the subsequent calculations for relative quantification and calculating assay variability.

Amplification efficiencies are also tested to detect anomalous samples within groups (outliers) and differences between experimental groups (amplification equivalence).

For additional details see *Peirson, Butler and Foster (2003)*.

DART-PCR converts raw data into R_0 values, based upon the theory that fluorescence is proportional to DNA concentration. Data must then be normalised by dividing target gene R_0 by the internal control R_0 to give a normalised expression value. Unfortunately, this normalisation is difficult to automate, as it is dependent upon exact experimental design (eg: controls may be run on separate plates).

$$\text{Normalised } R_0 = \frac{\text{target gene } R_0}{\text{internal control } R_0}$$

Different genes should be analysed independently

Basic Use

Basic operation may be summarised as follows:

1. Import data into DART-PCR workbook
2. Assign experimental groups and position (eg: controls = 1, treated = 2)
3. Ensure amplification efficiency is comparable
4. Enter amplification efficiency and threshold to be used for analysis

Only cells coloured blue allow user input - other cells are locked and contain outputs

1. Import data

Simply export the data in a fluorescence versus cycle format (normalised to passive reference if present), paste this data into the *Raw Data* sheet with the first fluorescence value for sample 1 at cycle 1 in cell B2. DART will then determine the amplification efficiency for each sample.

The DART workbook extracts the data from specific columns on the Raw Data sheet, and as such, if columns are missing from the data, these values will be misassigned. Conduct modifications on raw data before pasting into DART.

2. Assign experimental groups and position

Reactions should be set up in triplicate as this allows intra-assay variation to be calculated for both amplification efficiency and relative expression value (R_0).

Assign each experimental group a number (for example, control = 1 and treatment = 2). Analyse each gene separately - for example analyse target gene control and treatment, then analyse internal control control and treatment.

Triplicates are activated by placing a group number (1-6) in the *Sample setup grid*. Up to six different experimental groups can be assigned in this way, and data is summarised from this assignment. The mean Ct is then displayed in the *Ct Summary* box, and the first sample in every triplicate is shown on the DART sheet as an amplification plot.

3. Ensure amplification efficiency is comparable

Once assigned, the amplification efficiency will be calculated for all samples under analysis, and ANOVA is then used to test for outliers and for differences in amplification efficiency between groups. R_0 values are calculated (**Yellow box**) and a fold change based upon these values (and the calibrator group) will be shown. *At this stage, these are individual corrections and may not be suitable.*

If outliers exist, they may be identified and may require exclusion.

If differences exist between groups, each group should be analysed using its own efficiency.

If no differences are apparent, then the mean amplification efficiency should be used.

4. Enter amplification efficiency and threshold to be used for analysis

The primary user input is the amplification *Efficiency* to use (use of amplification efficiency of 1.00 will give identical results to the $2^{-\Delta\Delta Ct}$ method) and the **threshold** to calculate Ct from. If no value is entered into these boxes, then individual corrections will be applied, which are likely to yield systematic errors increasing assay noise (see paper for details).

If samples demonstrate comparable amplification efficiency, the mean efficiency may be used.

If differences exist between groups, different efficiency values may be used. Care should be exercised when applying individual corrections - small differences in efficiency may have a large impact on data.

We recommend using a *Threshold* based upon the mean M value ensures that all samples are in the linear phase of amplification, though if noise is apparent from the amplification plots this may be increased.

The expression summaries provided (including graph) are only for the gene currently under analysis to provide an idea of the differences between samples. The R_0 values should be normalised to give a final relative expression value.

Finally, the coefficient of variance is shown for R_0 and efficiency as a measure of intra-assay variance. This is continuously updated, and will be much higher when using individual corrections.

Additional Options

Midpoint

If blank, then the midpoint will be determined for every sample based upon the raw data. If a value is entered here, then the same midpoint will be used for all samples. This may produce differences where amplification efficiency declines at different rates, and is included to allow flexibility when fitting is problematic.

Minimum Points

The minimum number of points which will be accepted may be changed. The default is 3, though increasing this selection criteria may improve accuracy. If the range is not also increased then few samples may return amplification efficiency values.

Range

The range around the midpoint to be used to determine reaction efficiency may be changed. The default is set at 10, but for samples exhibiting a long linear phase, this range may be increased. If the range is too large, data points affected by noise or plateauing may be included.

Noise Factor

This factor was included to allow flexibility when dealing with noisy amplification plots, and this value changes the importance of the background noise factor. This is primarily of use when background corrections lead to deflections of the amplification plot baseline, and should only be used as a last measure.

Calibrate to

This value determines which group the fold change is calibrated relative to. The default is 1, indicating that the control group should be designated group 1.

Final Notes

The DART data setup is based upon running reactions in triplicate to enable assay variation to be calculated. Whilst this practice is recommended, users who are running large sample sets may wish to use individual values, and future updates may be set up to accommodate these changes.

We are continually updating this workbook and welcome input and discussion to enable a more accurate and user friendly means of analysing real-time PCR data.

Additional options for data analysis may be included in the future.

Example of Use

The following is provided in addition to the DART-PCR workbook to provide an example of data handling

The raw data contained within this workbook contains the experimental data described in the manuscript.

The setup of this data is as follows:

	1,2,3	4,5,6	7,8,9	10,11,12
A	1	1	1	1
B	2	2	2	2
C	3	3	3	3
D	4	4	4	4
E				
F				
G				
H				

Group

- 1 c-fos wildtype
- 2 c-fos rd/rd cl
- 3 B-actin wildtype
- 4 B-actin rd/rd cl

- 1 Copy the data from this sheet (rows 1-41) and paste special (values) into the Raw Data sheet of the DART-PCR workbook
- 2 Switch to the DART-PCR worksheet
- 3 Enter the group names (optional)
- 4 Assign well positions for c-fos by entering 1 into row A of the sample setup (wildtype) and 2 into row B (rd/rd cl)

	1,2,3	4,5,6	7,8,9	10,11,12
A	1	1	1	1
B	2	2	2	2
C				
D				
E				
F				
G				
H				

- 5 R0 values and at this stage are individually corrected and the Expression CV will be high due to systematic error:
- 6 Confirm amplification efficiency is comparable and if so, enter mean efficiency (0.846 for c-fos data)
- 7 Enter a threshold to use for analysis (the mean midpoint value is recommended to ensure all samples are in the linear phase - 0.063 for c-fos)
- 8 Check R0 values - assay variability should be much reduced compared to individual correction:

c-fos R₀ values

wildtype 5,278E-09 6,186E-09 4,894E-09 5,487E-09
rd/rd cl 1,381E-09 1,012E-09 1,241E-09 1,072E-09

- 9 The c-fos expression data relative to the wildtype (calibrator group = 1) will be shown. This is not normalised to B-actin
- 10 If the calibrator is changed to 2, the graph and summary will show expression relative to the rd/rd cl
- 11 Copy the Expression (R₀) data to a separate sheet, or to the User calculation section.
- 12 Repeat steps 4-10 for the B-actin data (mean efficiency = 0.874 and mean M = 0.069).

	1,2,3	4,5,6	7,8,9	10,11,12
A				
B				
C	3	3	3	3
D	4	4	4	4
E				
F				
G				
H				

- 13 Graph will be blank as still normalising to group 1. To see data summary, change calibrator to 3 to normalise to wildtype B-actin
- 14 B-actin levels should be comparable

B-actin R_0 values

wildtype 1,908E-07 2,301E-07 2,096E-07 1,923E-07
rd/rd cl 2,108E-07 1,932E-07 2,287E-07 2,135E-07

- 15 Normalise each sample by dividing c-fos expression by B-actin expression

Normalised Data (c-fos/B-actin)

wildtype 0,027664 0,0268912 0,0233472 0,0285263
rd/rd cl 0,0065524 0,0052418 0,0054239 0,0050237

- 16 Calculate the mean and standard deviation of these values as normal using Excel functions AVERAGE and STDEV

	mean	st dev
<i>wildtype</i>	2,66E-02	2,27E-03
<i>rd/rd cl</i>	5,56E-03	6,81E-04

- 17 To convert data to a fold change relative to wildtype, divide mean (and SD) by wildtype mean

FOLD	mean	st dev
<i>wildtype</i>	1,000	0,085
<i>rd/rd cl</i>	0,209	0,026

In comparison, individual corrections give:

c-fos R_0 values

wildtype 4,362E-09 6,743E-09 5,171E-09 1,002E-08
rd/rd cl 4,023E-09 9,393E-10 1,443E-09 7,271E-10

B-actin R_0 values

wildtype 2,093E-07 1,881E-07 3,253E-07 1,992E-07
rd/rd cl 1,913E-07 2,253E-07 2,149E-07 2,692E-07

Normalise:

Normalised Data

wildtype 0,0208386 0,0358451 0,0158962 0,0502812
rd/rd cl 0,0210309 0,004169 0,0067138 0,0027007

Summarise:

	mean	st dev
<i>wildtype</i>	3,07E-02	1,56E-02
<i>rd/rd cl</i>	8,65E-03	8,42E-03

Fold:

FOLD	mean	st dev
<i>wildtype</i>	1,000	0,507
<i>rd/rd cl</i>	0,282	0,274

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Experiment

Sample Setup

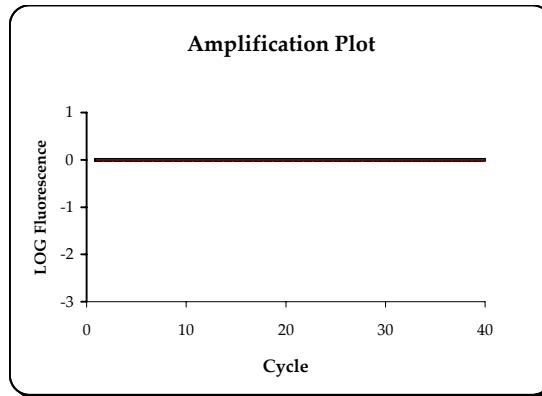
	1	4	7	10
A				
B				
C				
D				
E				
F				
G				
H				

Ct Summary

	1	4	7	10
A				
B				
C				
D				
E				
F				
G				
H				

EXPERIMENTAL GROUPS

Group 1	
Group 2	
Group 3	
Group 4	
Group 5	
Group 6	



Linear Phase Determination

ESSENTIAL PARAMETERS

Efficiency	
Threshold	

ADDITIONAL OPTIONS

Midpoint	
Minimum points	3
Range	10
Noise factor	1,0
Calibrate to	1

	MEAN	SD
Mean Efficiency		
Mean Midpoint		
Mean points in LP		
<i>p-value</i>		
Between groups:		

Results

Efficiency Results

	1	4	7	10
A				
B				
C				
D				
E				
F				
G				
H				

Experimental Group Efficiency

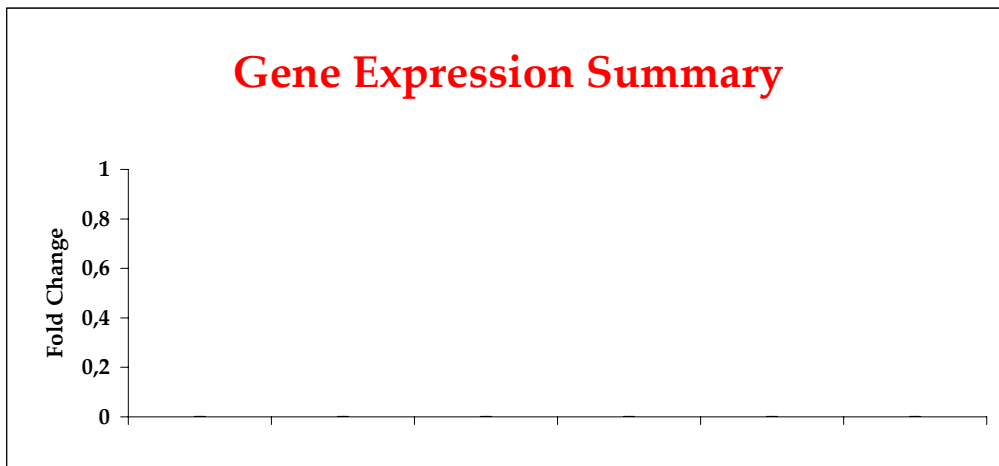
Sample	E	SD	p-value
1			
2			
3			
4			
5			
6			

Expression (R_0)

	1	4	7	10
A				
B				
C				
D				
E				
F				
G				
H				

Fold Change

Group	Fold	SD
1		
2		
3		
4		
5		
6		



Expression CV

	1	4	7	10
A				
B				
C				
D				
E				
F				
G				
H				

Mean CV

Efficiency CV

	1	4	7	10
A				
B				
C				
D				
E				
F				
G				
H				

Mean CV

Additional User Calculations