Determination of amplification efficiency
by dilution and continuous monitoring

R. Higuchi, 11/92

It is difficult to determine the replication efficiency during the early cycles of PCR directly, as it is difficult to accurately measure such small amounts of DNA. The continuous fluorescence monitoring of amplifications, however, allows a simple, indirect measurement based on the amplification of a series of dilutions of the original DNA template. As shown below, no information is needed for this measurement other than the magnitudes of the dilutions and the number of additional cycles necessary to bring all the dilutions up to the same fluorescence level. It is not necessary to know the actual number of PCR products made, or to precisely know the starting number of template molecules. It is not necessary to be in the log phase of PCR when the measurement is made. This analysis makes two main assumptions: 1) that an equivalent fluorescence value implies an equiivalent PCR product concentration, and 2) that the "profile" of amplifications be parallel for each dilution.

I. Relationship between amplification efficiency and the extra cycles needed to bring a template and its dilution to the same level of amplification

> Let: c_o = initial # of duplex DNA copies
>
> c_n = # of copies at cycle n
>
> c_{n+i} = # of copies at cycle n + i
> > (n and i can be non-integer)
>
> i = number of extra cycles needed in log phase
> > such that for a dilution of c_o, $c_n = c_{n+i}$
>
> z = 1 + fractional efficiency of amplification;
> > (*i.e.,* 99% efficiency = .99)
>
> k = dilution factor of C_o
> > (*i.e.,* 1:9 dilution = 10-fold; dilution factor = 10)

The equation describing the log phase of PCR is:

$$c_n = c_o z^n$$

If, in log phase, $c_n = c_{n+i}$, then:

$$c_o z^n = (c_o \div k) z^{n+i}$$
$$k z^n = z^{n+i}$$
$$k = z^{n+i} \div z^n$$
$$k = z^i$$
$$\log k = i \log z$$
$$\log z = 1 \div i * \log k$$
$$\text{or, } z = k^{1/i}$$

So if the dilution factor is two and it takes 1.2 extra cycles to catch-up:

$$z = 2^{1/1.2} = 1.67; \text{ or the efficiency is } 67\%$$

If one has a series of multiple dilutions, a plot of the log of the dilution factor, k, vs. i would be an equation of the form:

$$\log k = \log z * i$$

this is an equation of a line whose slope = log z,

$$\therefore \quad z = 10^{slope}$$

II. Validity outside of log phase

I have shown that if $c_n = c_{n+i}$ in log phase, the above is true. However, as long as the amplification profiles are parallel, the number of cycles offset between dilutions, once established, remains constant, even as the amplifiications approach plateau. For example, in the hypothetical amplification profiles shown below, the two amplifications are in log phase up to 32 copies, and diminiish in efficiency until they plateau at 192 copies. The separation between the two profiles remains constant even out of log phase. This separation is in fact determined by the initial efficiency of the

amplification of the dilution as it catches up to c_o (see indicated region in diagram). As long as the profiles of the two reactions are identical past this point, the separation between them does not change until they are absolutely at plateau. Thus, measurements of z should be possible even when reactions are not in log phase, and the efficiency that is being measured is that of the initial cycles of the amplification of the dilution.
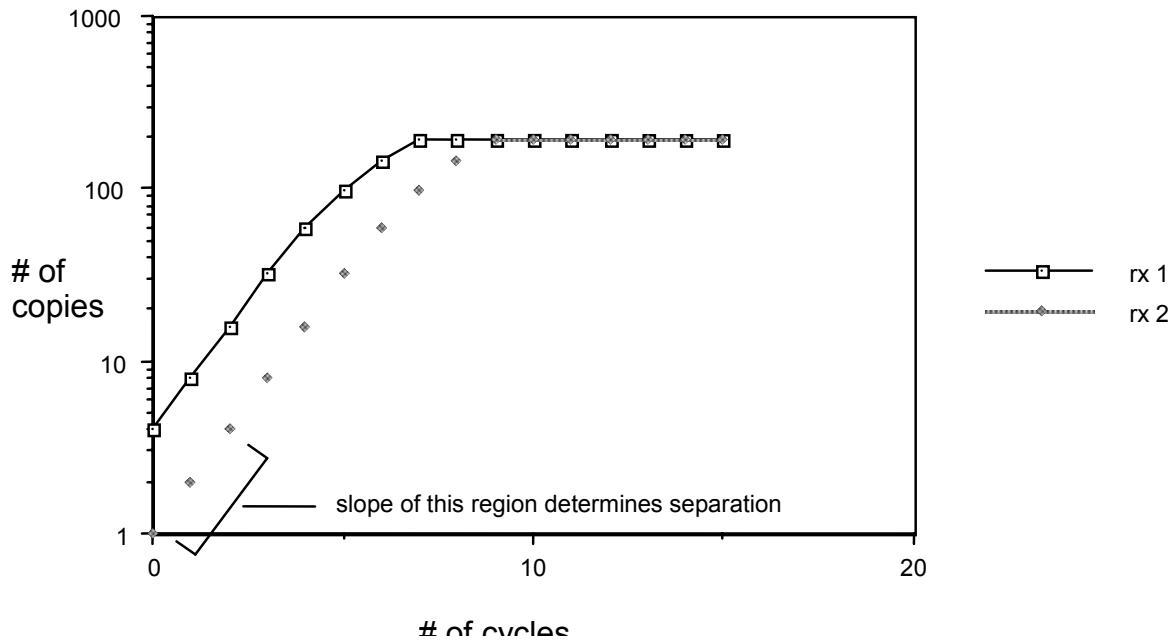

III. Example

The diagram below shows part of a continuous fluorescence monitoring of amplifications of HIV DNA done on serial, 10-fold dilutions of HIV template. The first profile, on the left was begun with 10^8 copies, and the second, 10^7, the third 10^6, etc. We can fairly safely assume that at a given fluorescence level, say, 145, $c_n = c_{n+i}$. The number of cycles it takes to reach a fluorescence of 145 can be read off the chart,, and are 20.6, 23.8, 27.3, 30.4, 33.7, and 36.6, respectively.
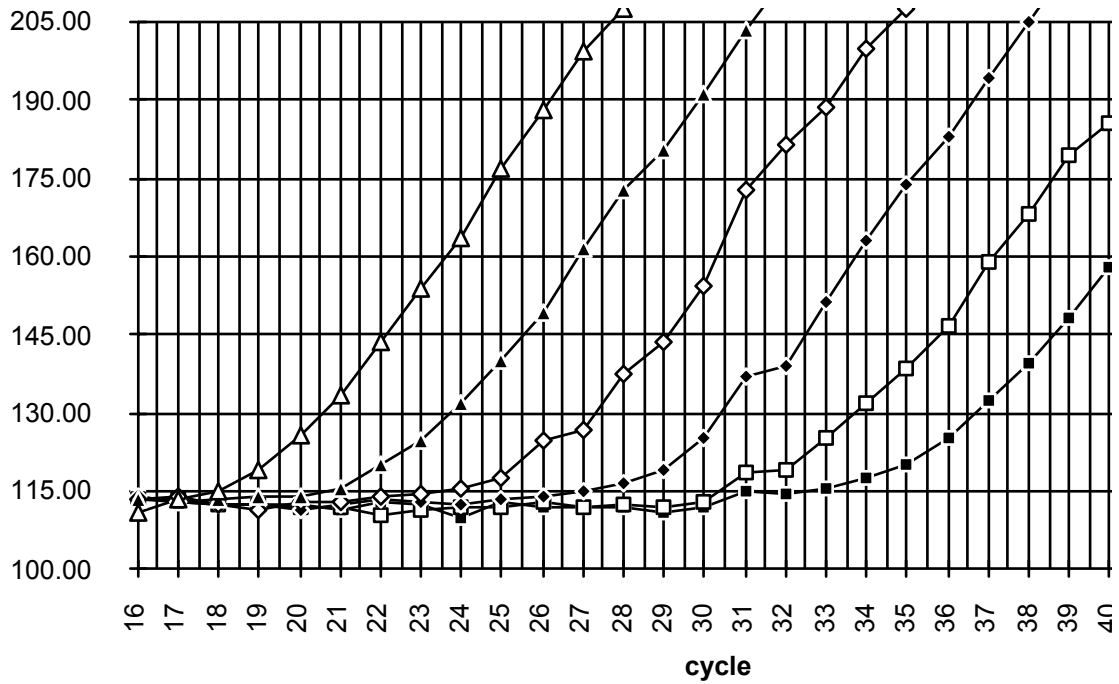
Plotting the log of the dilution factor applied vs. the number of cycles needed to reach a fluorescence of 145, one obtains a chart as shown below:

log dilution = - 6.6568 + 0.29924(cycle#)   R^2 = 0.999
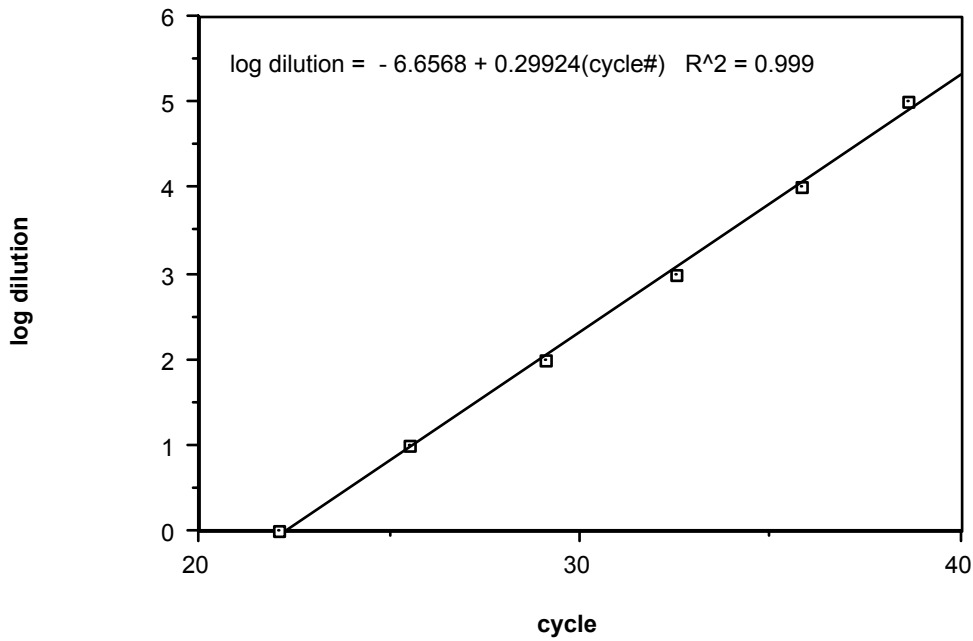
The equation in the graph is the regression-fitted line to the data points. The slope of the line = .29924. By the relationships derived in the first section, $z = 10^{.29924} = 1.99$. Thus the efficiency of the reaction (at below about 10^8 copies) is determined to be about 99%. For comparison, these analyses can be repeated for different fluorescence values. The regression lines, z values, and efficiencies for additional fluorescence levels are shown below:

Fluor. 130: log dilution = - 6.4104 + 0.31011(cycle#)   R^2 = 0.999
$10^{.31011}$ = 2.042; **effic. = 104%**

Fluor. 145: log dilution = - 6.6568 + 0.29924(cycle#)   R^2 = 0.999
$10^{.29924}$ = 1.991; **effic. = 99%**

Fluor. 160: log dilution = - 7.0955 + 0.30033(cycle#)   R^2 = 1.000
$10^{.30033}$ = 1.996; **effic. = 100%**

Fluor. 175: log dilution = - 7.3564 + 0.29628(cycle#)   R^2 = 0.999
$10^{.29628}$ = 1.978; **effic. = 98%**

Fluor. 190: log dilution = - 7.6965 + 0.29258(cycle#)   R^2 = 0.999
$10^{.29258}$ = 1.961; **effic. = 96%**

Fluor. 205: log dilution = - 7.8310 + 0.28513(cycle#)   R^2 = 0.999
$10^{.28513}$ = 1.928; **effic. = 93%**

The average efficiency from these calculations = **98%** with a std. deviation of **4%**.