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# An equation for calculating the volumetric ratios required in a ligation reaction

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**Abstract** The ligation of two DNA fragments to create a new plasmid DNA molecule is a key reaction in molecular biology. Where the fragment lengths and concentrations are known, existing equations allow the desired relative molar ratio to be calculated, but this must then be related to the required volumes. Further calculations are then necessary if the maximum available volume is to consist of DNA solutions. The equation presented here allows the simple calculation of volumes of DNA solutions required to obtain a desired molar insert-to-vector ratio, and these can comprise all of the available volume in a ligation if required, thus maximising the yield of the recombinant plasmid.

## Introduction

The ligation reaction is of fundamental importance to molecular biology by enabling the joining of vector and insert DNA fragments to create new plasmid DNA molecules. It is important to obtain the correct ratio of insert to vector for optimum recombinant plasmid generation and to reduce the formation of concatomeric fragments, religated vectors, plasmid dimers and plasmids containing multiple inserts.

A ligation reaction is usually carried out in a total volume of 10–20 μl at 4–37°C (often at room temperature) using the enzyme T4 DNA ligase, and a buffer containing $Mg^{2+}$ and ATP (typically from a stock concentration of ten times the working volume). T4 DNA ligase catalyses the joining of adjacent duplex DNA termini by the formation of phosphodiester bonds using ATP as a cofactor and can ligate either blunt or compatible cohesive ends. In an optimum ligation reaction aimed at inserting a single insert fragment into a vector, insert and vector DNA concentrations that disfavour monomolecular ring formation or concatamerisation should be used (Dugaiczyk et al. 1975; Sambrook et al. 1989).

Existing ligation equations (e.g. Doyle and Miles 1996) only allow the molar ratios of insert and vector DNA to be calculated. As the length of a DNA molecule is directly proportional to its molecular weight, such equations offer no advantage over simply determining the ratio of fragment lengths. The equation described here allows the component volumes to be easily calculated given the DNA concentrations and fragment lengths, such that the chosen insert-to-vector molar ratio is achieved and the reaction can consist of DNA ligase, buffer, insert and vector DNA solutions only if required.

## Results

Using the equation in Fig. 1, the volume of the vector component required in the ligation is calculated first, then this is subtracted from the total DNA component of the reaction (*T*) to give the required insert volume. The value *T* represents the combined volume of vector and insert DNA solutions added to the ligation reaction (e.g. *T*=8 μl in a 10-μl reaction with 1 μl each of ligase and buffer). The lengths and concentrations of the DNA fragments must be inserted along with the desired insert-to-vector ratio.

The equation was tested to verify that the results generated for the predicted volumes did indeed give the correct molar ratios of DNA fragments (Table 1). Arbitrary values (input) were used to generate predicted volumes using the ligation equation (output). The moles of DNA were calculated to determine the insert-to-vector ratio in theoretical ligation reactions set up using the volumes generated by the equation (test).

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$$V_v = \frac{T}{\left(\frac{V_c \cdot I_l \cdot I_r}{I_c \cdot V_l}\right) + 1} \qquad I_v = T - V_v$$

**Fig. 1** An equation for determining the volumes required in a ligation reaction. Insert and vector parameters must both be in the same units (e.g. kilobases for length and micrograms per microlitre for concentration). $I_l$ Insert length, $V_l$ vector length, $I_c$ insert concentration, $V_c$ vector concentration, $I_r$ required insert-to-vector ratio, $T$ volume of total DNA solution component, $V_v$ vector volume, $I_v$ insert volume. The $I_r$ should be inserted as insert/vector (e.g. 2 for a two-fold insert excess, 0.5 for a two-fold vector excess)

## Discussion

This equation will assist the molecular biologist at the bench by allowing the quick and simple calculation of the ratios required in the ligation reaction whilst maximising the DNA concentration, thus increasing the probability of generating the required recombinant plasmid molecule. No experimental evidence is presented here, as the experimenter chooses the factors that influence the ligation reaction. This equation will not produce values that are necessarily better than those that could be determined by more extensive calculations, but it will greatly facilitate the process. The examples in Table 1 show that the volumes calculated using the equation generate molar insert-to-vector ratios that are the same as the chosen ratios.

The chosen ratio depends on factors such as the DNA termini having blunt or cohesive ends, whether or not the vector has been treated with alkaline phosphatase and the concentration of DNA fragments in the reaction. Assuming that all four termini in a ligation are complementary, the theoretical optimum insert-to-vector ratio would take into account the ratio of the concentration of all termini in the reaction (*i*) to the concentration of one end of a linear DNA molecule in the volume occupied by the other end of the same molecule (*j*) (Dugaiczyk et al. 1975). The value *j* is inversely proportional to DNA fragment length and independent of DNA concentration, so it will be fixed for any given ligation. The key variables are therefore the total DNA concentration and the insert-to-vector ratio. For cohesive termini, Sambrook et al. (1989) recommend a *j*:*i* of between 1:1 and 1:3 (20–60 ng $\mu l^{-1}$ for a cloning vector the size of pUC18) and an insert-to-vector ratio of 2:1 for maximum yield of useful recombinants.

If the DNA fragment concentrations available for a cohesive-end ligation are low, it may be advantageous for the insert and vector DNA to comprise the remaining volume that is not occupied by ligase and buffer so that it is not necessary to add water to obtain the final reaction volume (within the limits of optimum *j*:*i*). The resulting higher DNA concentration at the correct component ratio increases the probability that a given insert molecule will ligate with a vector molecule rather than self-ligating, thus increasing the number of recombinant plasmid molecules generated. This is desirable as transformation efficiency increases when transforming competent *Escherichia coli* cells with increasing numbers of plasmid molecules (Seidman et al. 1994). A small ligation volume must be used to directly transform electrocompetent cells, as the presence of salts from the ligation buffer can cause arcing during electroporation, resulting in low transformation efficiencies (Hanahan et al. 1991). Therefore a high DNA concentration can be advantageous.

Blunt-end ligations occur with a much lower efficiency than cohesive-end ligations, and in a single ligation

**Table 1** Testing the ligation equation. Arbitrary values for insert and vector concentrations and volumes, DNA fragment lengths, total DNA volume and insert-to-vector ratio were chosen (example A input) and the ligation equation was used to calculate predicted volumes generating the chosen insert-to-vector ratio (output). Single input parameters were altered in examples B–H. The number of femtomoles in the predicted vector and insert volumes ($I_{mol}$ and $V_{mol}$) were calculated by dividing the mass of DNA in the predicted volumes by the mass of 1 mol of the DNA fragment by using the formula: (volume×concentration)/(650×length in base pairs), where 650 g is the mean mass of 1 mol of a base pair. $I_l$ Insert length, $V_l$ vector length, $I_c$ insert concentration, $V_c$ vector concentration, $T$ volume of total DNA solution component, $V_v$ vector volume, $I_v$ insert volume. The required ($I_r$) and actual ($I_{mol}/V_{mol}$) insert-to-vector ratios are given

| | | Example | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | A | B | C | D | E | F | G | H |
| Input | $I_c$ (ng $\mu l^{-1}$) | 20 | 20 | 20 | 80[a] | 20 | 20 | 20 | 20 |
| | $V_c$ (ng $\mu l^{-1}$) | 50 | 50 | 50 | 50 | 90[a] | 50 | 50 | 50 |
| | $I_l$ (kb) | 4 | 4 | 4 | 4 | 4 | 9[a] | 4 | 4 |
| | $V_l$ (kb) | 3 | 3 | 3 | 3 | 3 | 3 | 5[a] | 3 |
| | $T$ (µl) | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 16[a] |
| | $I_r$ | 2 | 1[a] | 0.5[a] | 2 | 2 | 2 | 2 | 2 |
| Output | $I_v$(µl) | 6.96 | 6.15 | 5.00 | 5.00 | 7.38 | 7.50 | 6.40 | 13.91 |
| | $V_v$(µl) | 1.04 | 1.85 | 3.00 | 3.00 | 0.62 | 0.50 | 1.60 | 2.09 |
| Test | $I_{mol}$ (fmol) | 53.51 | 47.34 | 38.46 | 153.85 | 56.80 | 25.64 | 49.23 | 107.02 |
| | $V_{mol}$ (fmol) | 26.76 | 47.34 | 76.92 | 76.92 | 28.40 | 12.82 | 24.62 | 53.51 |
| | $I_{mol}/V_{mol}$ | 2 | 1 | 0.5 | 2 | 2 | 2 | 2 | 2 |

[a]Single input parameters altered in examples B–H

reaction the optimum total DNA concentration is 1–5 ng $\mu l^{-1}$ with a significant reduction in transformation efficiency at concentrations over 10 ng $\mu l^{-1}$ (Bercovich et al. 1992). However, transformation efficiencies of blunt-end ligations can be improved by using a two-step method which favours the initial intermolecular ligation of linear insert to vector with a high DNA concentration, then a dilution results in the low concentration that favours the intramolecular ligation resulting in circularisation (Damak and Bullock 1983).

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