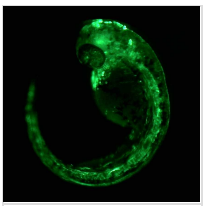

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Multisite Gateway Calculations: Excel spreadsheet

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Multisite Gateway cloning provides a modular tool to assemble plasmid vectors suitable for numerous applications. The assembly of Multisite Gateway plasmids requires the correct ratios of three entry vectors and the destination backbone to favor correct recombination by Gateway LR Clonase II Plus. While simple, the calculation of recombination mixes is a reoccurring task when using Multisite Gateway that benefits from automation and proper documentation. This protocol provides a simple Excel-based spreadsheet to simplify the involved calculations based on providing individual vector lengths and working stock concentrations. Using this Multisite Gateway calculations spreadsheet supports correct reaction mix assembly, scaling of cloning reactions, cloning documentation, and troubleshooting.

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Multisite Gateway cloning provides a modular tool to assemble plasmid vectors suitable for numerous applications. The assembly of Multisite Gateway plasmids requires the correct ratios of three entry vectors and the destination backbone to favor correct recombination by Gateway LR Clonase II Plus. While simple, the calculation of recombination mixes is a reoccurring task when using Multisite Gateway that benefits from automation and proper documentation. This protocol provides a simple Excel-based spreadsheet to simplify the involved calculations based on providing individual vector lengths and working stock concentrations. Using this Multisite Gateway calculations spreadsheet supports correct reaction mix assembly, scaling of cloning reactions, cloning documentation, and troubleshooting.

Multisite Gateway calculations using a simple Excel spreadsheet

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Multisite Gateway (1) is a widely used cloning method based on the widespread availability of individual component vectors through repositories such as AddGene (www.addgene.org) and a

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multitude of published plasmids from individual laboratories or organizations working on diverse models. For zebrafish, in particular the Tol2 Kit vectors for transgenesis have introduced a multitude of zebrafish labs to Gateway cloning, stimulating vector exchange and simple, modular assembly of new transgenesis tools (2).

Based on the recombination reaction catalyzed by Gateway LR Clonase II Plus, Multisite Gateway cloning incorporates Entry vectors with inserts flanked by dedicated repeats (commonly called Gateway repeats) into a final Destination vector. In their most-applied form, such multi-vector assemblies consist of 1) a 5' entry vector harboring a gene-regulatory element or promoter; 2) a middle entry vector harboring an ORF encoding a fluorescent protein or other factors of interest; 3) a 3' vector harboring a polyadenylation signal in a untranslated trailed sequence; and 4) a Destination backbone with suitable elements for transgenesis, bacterial selection, and more. A multitude of modular assemblies are possible beyond such basic expression constructs.

The successful assembly by recombination of all these four plasmids requires a well-balanced ratio of the individual parts in the reaction: standard reactions combine 10 femtomole (fmol) of each Entry vector with 20 fmol of the Destination backbone vector together with LR Clonase II Plus in a 10 microliter (µl) reaction volume. This reaction is incubated for 16+ hours at 25 degrees Celsius before short Proteinase K treatment and subsequent transformation into suitable competent bacteria for selection and propagation (as per manufacturer's protocol in Ref. 1). The original manufacturer's protocol describes 10 µl reactions, yet scaling down to 5 µl reactions has proven practical and economical (3,4).

While seemingly mundane, a common challenge in assembling Multisite Gateway reactions is the proper calculation of equal fmol amounts of each involved vector in the reaction tube. While the involved calculations are simple, having a means to rapidly calculate reaction mixes a) provides an additional safeguard against calculation errors; b) enables scaling up of reaction mix calculations to tackle several clonings at once; c) document the involved calculations for trouble-shooting, lab book documentation, and publications.

The final goal is to assemble a 5 µl reaction consisting of 4 µl total solution containing all four vectors in ddH2O plus 1 µ of LR Clonase II Plus.
Assuming the classic 660 g/mol for a DNA base pair, the calculations to achieve a final 10 fmol amount per vector in a 5 µl Multisite Gateway reaction is:

1. *[total length of vector] bp x 10 fmol x (660/10^6) = [total vector amount] ng*
2. *([total vector amount] ng / [concentration vector stock] ng/µl) / 2 (for 5 µl half-reactions) = µl for 10 fmol*

To reach a total of 4 µl final vector solution, calculate:
3. *4 µl - ([volume 5' vector 1] + volume middle vector] + [volume 3' vector] + [volume backbone vector])*
= µl ddH2O to reach 4 µl reaction mix before adding LR Clonase II Plus

These simple, yet repetitive calculations are combined here in an Excel-based spreadsheet that includes annotation fields to keep track of used vectors. The spreadsheet has pre-formatted calculation sections for two assemblies, and can easily be expanded by copy/paste to accommodate

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more. We have been using this spreadsheet since 2008 to reproducibly assemble Multisite Gateway vectors for zebrafish transgenesis (3,4). Rounding of the calculated numbers depends on individual Excel settings and, at the bench, should be assumed within practical reason for pipetting.

Saving a dedicated spreadsheet for each newly assembled vector or vector group is recommended as best practice, and to additionally document so in your personal lab book (hard copy or digital). In the lab, we commonly print the spreadsheet calculations for documentation purposes.

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	**MultisiteGateway Cloning Spread Sheet**											Christian Mosimann, 11/19/2009	
2	*vector name*												
3			**bp total**	**fmol**	**total**				**plasmid concentration**			**use**	
4													
5	**5' Element**	*pCM293*	*9027*	10	59.5782	**ng**			40	**ng/ul**		0.74473	**ul**
6	**ORF**	*Tol2 kit 383*	*5631*	10	37.1646	**ng**			40	**ng/ul**		0.46456	**ul**
7	**3' UTR**	*Tol2 kit 302*	*2838*	10	18.7308	**ng**			20	**ng/ul**		0.46827	**ul**
8	**Backbone**	*pCM268*	*5085*	20	67.122	**ng**			79	**ng/ul**		0.42482	**ul**
9													
10									ddH2O			1.89762	**ul**
11													
12									add 1ul LR Clonase II PLUS			**5**	**ul total**
13													
14	*vector name*												
15			**bp total**	**fmol**	**total**				**plasmid concentration**			**use**	
16													
17	**5' Element**	*pCM202*	*6825*	10	45.045	**ng**			25	**ng/ul**		0.9009	**ul**
18	**ORF**	*Tol2 kit 383*	*5631*	10	37.1646	**ng**			40	**ng/ul**		0.46456	**ul**
19	**3' UTR**	*Tol2 kit 302*	*2838*	10	18.7308	**ng**			20	**ng/ul**		0.46827	**ul**
20	**Backbone**	*pCM268*	*5085*	20	67.122	**ng**			79	**ng/ul**		0.42482	**ul**
21													
22									ddH2O			1.74145	**ul**
23													
24									add 1ul LR Clonase II PLUS			**5**	**ul total**
25													

Figure 1: Multisite Gateway Cloning Spread Sheet to calculate cloning reactions involving all necessary vectors at correct concentrations, final water volume, and enzyme.

The Multisite Gateway Calculation sheet is used as follows in a cloning workflow:

1. Fill in individual **vector names** in **column B**
2. Fill in individual total **vector size** in base pairs in **column C**
3. Fill in individual **vector stock concentrations** in **column I**
4. Double-check pipetting volumes are practical (i.e. not below 0.2 µl; working stocks 20-60 ng/µl)
5. Add 1 µl LR Clonase II Plus as last step (vortexed 2x for 2 sec), mix by flicking followed by quick spin
6. Incubate reactions at 25 degrees Celsius for 16 h+

General Multisite Gateway tips:

- Use clean minipreps, verified by 260/230 nm and 260/280 nm measurements; avoid kit elution buffers
- Use ddH2O to make working stocks for gateway
- Always use clean, fresh ddH2O or TE
- Always vortex the Clonase 2x for 2 seconds to properly mix the stock (crucial)
- Mix the reaction once assembled
- Do not use too concentrated vector stock solution, working stocks in the range of 20-60 ng/µl work well

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Download the spreadsheet here:

MultisiteGateway Calculations v1 Feb2022.xlsx

Taken together, this simple spreadsheet facilitates the reproducible and documented assembly of Multisite Gateway reactions with three entry vectors and their targeted destination vector.

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