

Gateway Cloning

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Sample to Insight -

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Gateway Cloning

This tutorial will show you how to do in silico Gateway®Cloning in a CLC Workbench. While you can perform both standard (1 fragment) and MultiSite Gateway Cloning, we focus here on the 1-fragment cloning approach. We provide tips to help you move onto MultiSite Gateway Cloning easily.

For information on the Gateway Cloning system please visit: https://www.thermofisher. com/us/en/home/life-science/cloning/gateway-cloning.html.

This tutorial covers the following:

- Import Gateway Cloning vectors into your Workbench.
- Add attB sites to the fragment of interest, as would be done in the lab using PCR.
- Construct an entry vector by an attB/attP recombination event (BP reaction) in which the attB-flanked fragment is exchanged for the attP-flanked sequence of the chosen donor vector.
- Construct the final expression vector by an attL/attR recombination event (LR reaction) in which the target fragment of the entry vector is exchanged for the attR-flanked sequence of a chosen destination vector.

Importing Gateway Cloning vectors

Donor and destination vectors can be imported as *txt files. The sequences we need for this tutorial can be found at:

- https://tools.thermofisher.com/content/sfs/vectors/pdonr221_seq.txt
- https://tools.thermofisher.com/content/sfs/vectors/pdest14_seq.txt

You can now save the vector sequences into the Workbench:

- 1. Use the **New** (]-) button and select the option **Sequence**.
- 2. A Create Sequence window opens (figure 1)

Enter the name of the Gateway vector and paste the TXT sequence you copied on the Thermofisher website. Check the option "Circular" and click **OK**.

3. Save the vector in the Navigation Area (for example in a new folder called "Gateway Cloning").

The vector sequences should now be imported into your new folder.

For further information about the vectors for MultiSite Gateway Cloning, please refer to Thermo Fisher Scientific's web site that can be found here https://www.thermofisher.com/us/ en/home/life-science/cloning/gateway-cloning/multisite-gateway-technology. html.



	Enter Sequence Data		
Enter Sequence Data	Name: pdest1	14	
	Common name:		
	Latin name:		1
	Type: X 🍥	DNA	
	XOC (1)	RNA	
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	Sequence (required)		64
	GCCCACCGGAAGGAG	CTGACTGGGTTGAAGGCTCTCAAGGGCATCGGTCGATCGA	
	GACTCCTGCATTAGG	AAGCAGCCCAGTAGTAGGTTGAGGCCGTTGAGCACCGCCGCCGCAAGGAATGGTG	
	CATGCAAGGAGATGG	CGCCCAACAGTCCCCCGGCCACGGGGCCTGCCACCATACCCACGCCGAAACAAGC	
	GCTCATGAGCCCGAA	GTGGCGAGCCCGATCTTCCCCATCGGTGATGTCGGCGATATAGGCGCCAGCAACC	
	GCACCTGTGGCGCCG	GTGATGCCGGCCACGATGCGTCCGGCGTAGAGGATCG	

Figure 1: Creating a circular sequence from a text file.

Adding attB sites

This step mimics the lab PCR procedure of adding attB sites to the target fragment making it eligible for recombination into the donor vector. The output can also include a set of primers, although we do not do that in this tutorial.

Our target fragment is the coding sequence (CDS) of ATP8a1, the mRNA of which is found in the standard example data you can download into the Workbench. If you do not already have the example data go to the **Help** menu in the Workbench, and choose to **Import Example Data**.

- 1. Extract the CDS sequence from the ATP8a1 mRNA file. To do this,
 - Double click on the sequence object called **ATP8a1 mRNA**. This opens it in the viewing area of the Workbench.
 - Right-click the CDS annotation, which by default will be colored in yellow, and chose **Open annotation in New View**
 - Save the new data object that has just opened in the new view. There are different ways to do this. Two options are:
 - (a) Right click on the tab at the top of the new view, and choose Save As... from the menu that appears, or
 - (b) Move the cursor over the tab at the top of the new view, press the left mouse button. Keeping the mouse button down, drag the tab over into a folder in the **Navigation Area** of the Workbench. The data will then be saved into that folder.
 - (c) Rename the CDS sequence to **ATP8a1 CDS** directly in the Navigation Area by doing a slow double-click on the name, or by selecting the element and choosing the menu item Edit | Rename.
- Now we can start to add the attB sites. To do this, start the Add attB Sites tool: Molecular Biology Tools | Cloning | Gateway Cloning | Add attB Sites (
- 3. In the wizard, select the ATP8a1 CDS fragment you just saved. When the wizard starts up, that fragment should be in the list of **Selected Elements**. If it is not, please select it in the wizard, and click on the right hand arrow to add it to the **Selected Elements** list. Click **Next**.



4. In the next step, choose the attB sites to be added to your fragment. This is shown in figure 2. The sites to choose depend on your choice of donor vector. We will be going with the default option of attB1 and attB2. This ensures compatibility with our downstream choice of donor vector, which will be **pDONR221**. Click on the button marked **Next**.

Gx Add attB Sites	X
 Choose where to run Select one or more fragments Select attB sites Specify primer additions Set primer extensions Result handling 	AttB sites AttB sites attB1 v AttB2 v
Help Reset	Previous Next Finish Cancel

Figure 2: Adding attB sites.

Note that if you have selected more than one fragment these will all be given the same combination of attB sites. If you wish to add different combination of sites you will have to run the tool once for each combination.

5. Next you will be given the option to insert additional elements downstream and upstream of the first and second attB site, respectively. We will add a Shine-Dalgarno site 5 prime of ATP8a1. Click in the field **Forward primer additions** and press on the keys Shift+F1 (for Mac: Shift + Fn + F1) to bring up a list of elements you can choose from. Use either the mouse or the up and down arrow keys and enter to select the Shine-Dalgarno site (figure 3).

Gx Add attB Sites	
 Choose where to run Select one or more fragments 	Set parameters Primer additions Forward primer additions
 Select attB sites Specify primer additions 	Shine-Dalgamo (AGGAGGT) Kozak + ATG (ACCATGG) Kozak (upstream of ATG) (ACC) Start codon (ATG) F His tag (CATCACCATCACCATCAC) Kozak + Peroxisomal targetting sequence (ACCATGGGCTGCGTGTGCTCCCGCGGCGGC) Lumio tag (TGTTGTCCTGGCTGTGC) TEV cleavage site (GACACATCACCATCAGGA) EK cleavage site (GACACTCACCATCAACATAA)
10 10 10 10 10 10 10 10 10 10 10 10 10 1	ACCCAGCTTICTTGTACAAAGIGGT CCCC attB2
? 5	← Previous → Next ✓ Finish X Cancel

Figure 3: Choose Shine-Dalgarno.

6. Insert 8 random nucleotides after the Shine-Dalgarno sequence. This is done by simply typing in the bases (figure 4). Doing this ensures that the spacing between the Shine-Dalgarno sequence and the initiation codon is correct.

You will find a preview of the final PCR product at the bottom of the wizard window. Any preset additional elements appear in green whereas the bases typed in manually are displayed in blue. Click **Next**.



× Add attB Sites	
1. Choose where to run	Set parameters Primer additions
2. Select one or more	Forward primer additions
fragments	AGGAGGTCCCAGCAT
3. Select attB sites	Press Shift + F1 for options Reverse primer additions
4. Specify primer additions	Press Shift + F1 for options
	Preview
CP2	attB1 Shine-Dalgamo
AND DATE OF THE	Sequence of interest
1 De Martine	ACCCAGCTITCTTGTACAAAGTGGT CCCC attB2
A PRAILING	
?	← Previous → Next ✓ Finish X Cancel

Figure 4: Add bases to ensure correct spacing between the Shine-Dalgarno sequence and the initiation codon.

7. In this step, you can choose the length of the template-specific part of your primers. This template-specific fragment is joined with the attB site and any additional elements you choose to make up the primer. Here, we will just keep the defaults (figure 5). Click on the button marked **Next**.

Gx Add attB Sites	
Choose where to run Select one or more fragments Select attB sites Specify primer additions Set primer extensions	Set parameters Primer extensions Forward primer extension 20 Reverse primer extension 20
? (%)	← Previous → Next ✓ Finish X Cancel

Figure 5: Set the appropriate primer lengths.

8. Choose to **Save** the results. Uncheck the **create primer list** option as we will not be needing this in this tutorial (figure 6). Click on the button marked **Next** and choose where to **Save** the output.

Open up the result **ATP8a1 CDS (attB1 attB2)** to have a look (figure 7). In the side panel called **Sequence Settings**, find the section called **Annotation types**. Click on it to expand it. Click on the box beside **RBS** to mark it, so that the Shine-Dalgarno ribosomal binding site annotation is displayed. Note also that you can see annotations for primer regions and the attB sites.

Creation of an entry vector

This step recombines the attB-flanked ATP8a1 into the donor vector, which is **pDONR221**, resembling the BP-reaction carried out in the lab.

1. Choose **Create Entry Clone** from under the Gateway Cloning menu:



1. Choose where to run	Result handling
2. Select one or more fragments	Output options
3. Select attB sites	Create primer list
 Specify primer additions Set primer extensions Result handling 	Result handling Open Save
The second secon	Log handling

Figure 6: Choose to save your results.

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	Forward primer	Atp8a1	*	Sequence Settings	
	attB1	Atp8a1	E	Sequence layout -	^
				Spacing	
				No spacing 🔹	
	20 40			💿 No wrap	
mRNA_Atp8a1 (attB1 attB2)	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGGAGGTACGTGTC	AATGCCGACCATGCGG		Auto wrap	
	Forward primer			○ Fixed wrap:	
	Atp8a1 Atp8a1			every 10000 residues	
				Every 10000 residues	
				Double stranded	
	80 80 100			Vumbers on sequences	=
mRNA_Atp8a1 (attB1 attB2)	AGGACAGTGTCGGAGATCCGCTCGCGCGCGGAAGGTTATGAGA	AGACAGATGATGTTTC		Relative to 1	
	_Atp8a1			Vumbers on plus strand	
	Atp8a1			✓ Lock numbers	
				Hide labels	
	120 140 180			V Lock labels	
mRNA_Atp8a1 (attB1 attB2)	AGAGAAGACCTCGCTGGCAGATCAGGAGGAAGTGAGAACCATC	TTCATCAACCAGCCTC		Sequence label	
	Atp8a1			Name	
	Alpoal			Annotation layout Annotation types	
	180 200 220				-
mRNA_Atn8a1 (attB1 attB2)					
	Ament			Position Next to sequence V	
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Figure 7: A view of the results: ATP8a1 CDS (attB1 attB2).

Tools | Molecular Biology Tools | Cloning | Gateway Cloning | Create Entry Clone (())

- In the dialog select the result that you generated earlier: ATP8a1 CDS (attB1 attB2) (figure 8). Then click on the button marked Next.
- 3. Click the **Browse button** () and find the Gateway Cloning folder where you previously saved your vector. Select donor vector **pDONR221** as shown in figure 9 and click **OK**. Click on the button labelled **Next**.

Note: The Workbench only checks the chosen entry clone for valid attP sites. It does **not** check that these are compatible with the attB sites present in the fragment. You need to make sure that a valid entry vector is used for the attB-fragment of interest. If the right combination of attB and attP sites is not found, no entry clones will be produced.

4. Choose to **Save** the result and click on the button labelled **Next** to be able to specify where to save the results to before clicking on the button labelled **Finish**.



G Create Entry Clone (BP)) Select att8-flanked fragments Navigation Area	Selected elements (1)
2. Select attB-flanked fragments	CLC_Data Gateway Cloning - % ATP8a1 mRNA_Atp8a1 - % ATP8a1 mRNA_Atp8a1 (att81 att82) - % BaculoDirect Linear DNA - % pAd_CMV_V5-DEST - % center search term> Batch	Image: State
?	← Previous	→ Next ✓ Finish X Cancel



1. Choose where to run	Set parameters Donor vectors				
2. Select attB-flanked fragments	pDONR221			<u>a</u>	
3. Select a dopor vector	Fragments				
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S C			*	pDONR221	
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			VOK X Cance	el	

Figure 9: Select the donor vector, which in this case is pDONR221.

Open the output **pDONR221 (ATP8a1 CDS (attB1 attB2))** to have a look at it. To see a view like the one shown in figure 10:

- Go to circular view (**O**).
- Check annotation type **RBS** in the side panel to get a better view. The attB/attP recombination event has resulted in the fragment of interest being inserted into the vector. The fragment is flanked by attL sites.

If several fragments were selected for input (using the batch option), the output would be one entry clone per fragment, all based on the same donor vector.

Creating an expression vector (LR)

This last step in a Gateway Cloning workflow mimics the lab LR recombination reaction. That is, the fragment of interest is recombined from the entry vector into a destination vector (the LR reaction) to create the final expression vector.

In this tutorial we are carrying out a standard, single fragment Gateway Cloning task. Thus we will choose only one entry clone as input during this step. For MultiSite Gateway Cloning, we would choose all the entry clones to be joined in the final expression vector. Note, that if you





Figure 10: View of the entry clone, consisting of the donor vector, pDONR221, with the fragment of interest inserted by attB/attP recombination.

do this, you need to ensure that the entry clones contain a valid combination of attL sites. For more information on this please have a look at the MultiSite Gateway explanation on the Thermo Fisher Scientific webpage:

https://www.thermofisher.com/us/en/home/life-science/cloning/gateway-cloning/ multisite-gateway-technology.html

The steps to create an expression clone are:

1. Choose **Create Expression Clone** from under the Gateway Cloning menu:

Tools | Cloning (()) Gateway Cloning | Create Expression Clone (())

- 2. In the dialog box select the entry clone you created in the previous step and click on the button marked **Next**.
- 3. In the next window, click the **Browse** button and navigate to the Gateway vector folder. Select the destination vector: **pDEST14** and click on the button labelled **OK** before clicking on the button labelled **Next** (figure 11).

Remember, the Workbench only checks for valid attR sites in the destination vectors. You need to ensure that the attL and attR sites are compatible. If not, no expression clone will be created.

4. In the next window choose to open the result. If you would like to also have a look at the byproduct, check the corresponding box. Click on the button labelled **Finish**.

The output is the result of the recombination between the attL and attR sites. The fragment of interest is now inserted in the expression vector flanked by attB sites as seen on figure 12.

This concludes the Gateway Cloning tutorial. Below is a section outlining the general steps you could take in the case of MultiSite Gateway Cloning.









Figure 12: View the finished expression vector.

Short suggestion for a MultiSite Gateway Workflow using 2 fragments

- 1. Choose two fragments, which you would like to have in the final vector.
- 2. Create the following fragments:
 - Add attB1 and attB5r sites to sequence of interest 1 -> sequence of interest 1 (attB1 attB5r)
 - Add attB5 and attB2 sites to sequence of interest 2 -> sequence of interest 2 (attB5 attB2)
- 3. Create two entry clones:
 - sequence of interest 1 (attB1 attB5r) + pDONR221-P1P5r
 - sequence of interest 2 (attB5 attB2) + pD0NR221-P5P2
- 4. Create the final expression vector by selecting:



- the first entry clone: pDONR221-P1P5r (fragment of interest 1(attB1 attB5r)), and
- the second entry clone: pDONR221-P5P2 (fragment of interest 2(attB5 attB2))
- the destination vector: pDEST14