



Tutorial

Gateway Cloning

September 15, 2016

— Sample to Insight —

Gateway Cloning

This tutorial will show you how to use the Workbench tool for in silico Gateway®Cloning. With this you can perform both standard (1 fragment) and multi-site Gateway Cloning. Here we focus on the 1-fragment cloning approach, but 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.


To be able to carry out the first steps of the tutorial (importing the .ma file) you first have to make sure that the **Vector NTI import** plugin is installed in your Workbench. For a description of how to install a plugin, please see the "Plugins" section in the CLC Genomics Workbench manual, which can be found here: <http://www.clcsupport.com/clcgenomicsworkbench/current/index.php?manual=Plugins.html>.

Importing Gateway Cloning vectors

To perform the in silico Gateway Cloning we first need to import donor and destination vectors. A file of these called "Gateway vectors.ma4" can be downloaded from the Invitrogen web site:

<http://tools.invitrogen.com/downloads/Gateway%20vectors.ma4>

Now, import the vector sequences into the Workbench.

1. Use the **standard import tool** () that you can find under File or by pressing Ctrl + I.
2. An Import wizard window opens. Select the Gateway vectors.ma4 file you just downloaded and click Next.
3. Select the location where you would like the vectors to be imported (in our case, a new folder called "Gateway Cloning") and click on the button labeled "Finish".

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](https://www.qiagen.com/en/home/life-science/cloning/gateway-cloning/multisite-gateway-technology.html).


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**.

Now:

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 coloured 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.
2. Now we can start to add the attB sites. To do this, start the **Add attB Sites** tool:

Toolbox | Molecular Biology Tools | Cloning and Restriction Sites | 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 on the button marked **Next**.
4. In the next step, choose the attB sites to be added to your fragment. This is shown in figure 1. 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**.

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

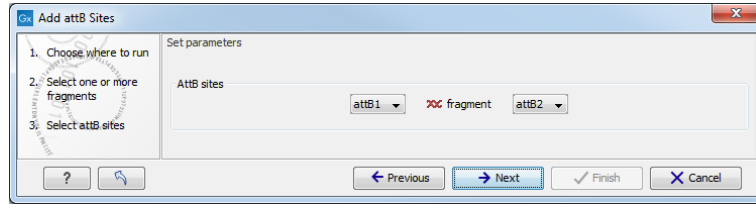


Figure 1: Adding attB sites.

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 2).

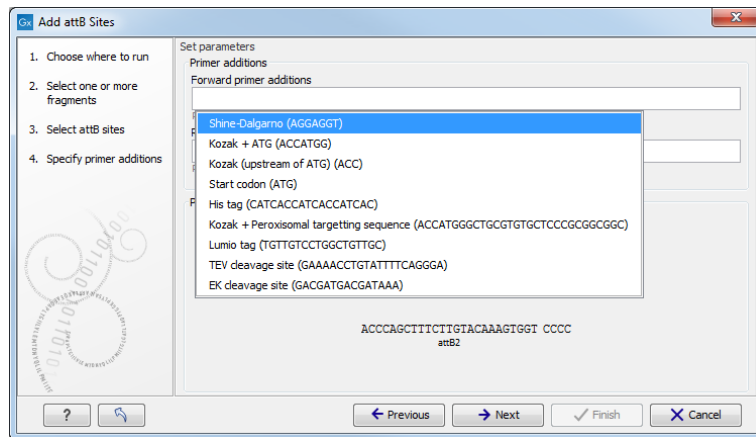


Figure 2: Choose Shine-Dalgarno.

6. Insert 8 random nucleotides after the Shine-Dalgarno sequence. This is done by simply typing in the bases (figure 3). Doing this ensures that the spacing between the Shine-Dalgarno sequence and the initiation codon is correct.

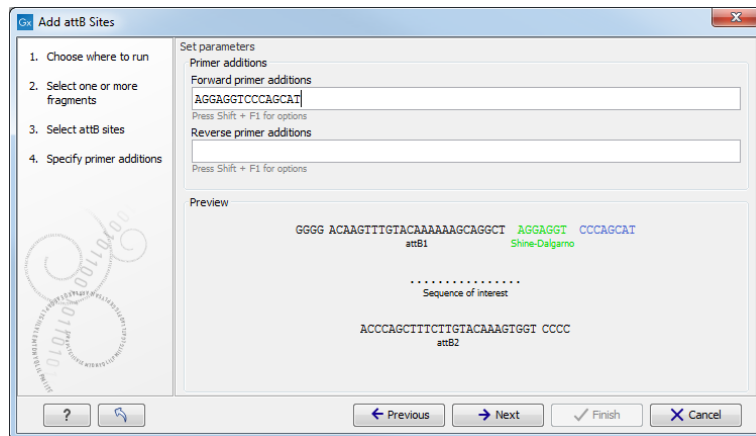


Figure 3: Add bases to ensure correct spacing between the Shine-Dalgarno sequence and the initiation codon.

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 on the button marked **Next**.

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 4). Click on the button marked **Next**.

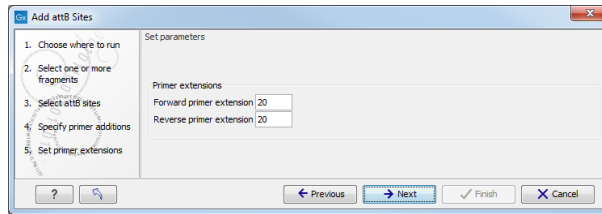


Figure 4: Set the appropriate primer lengths.

8. Choose to **Save** the results. Uncheck the **create primer list** option as we will not be needing this in this tutorial (figure 5). Click on the button marked **Next** and choose where to **Save** the output.

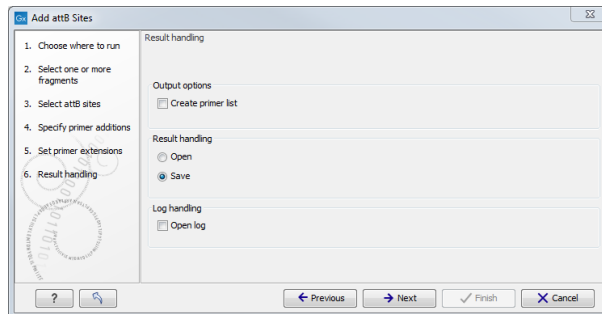




Figure 5: Choose to save your results.

Open up the result **ATP8a1 CDS (attB1 attB2)** to have a look (figure 6). 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:
Toolbox | Molecular Biology Tools | Cloning and Restriction Sites | Gateway Cloning | Create Entry Clone ()
2. In the dialog select the result that you generated earlier: **ATP8a1 CDS (attB1 attB2)** (figure 7). 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 8 and click on the button labelled **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.

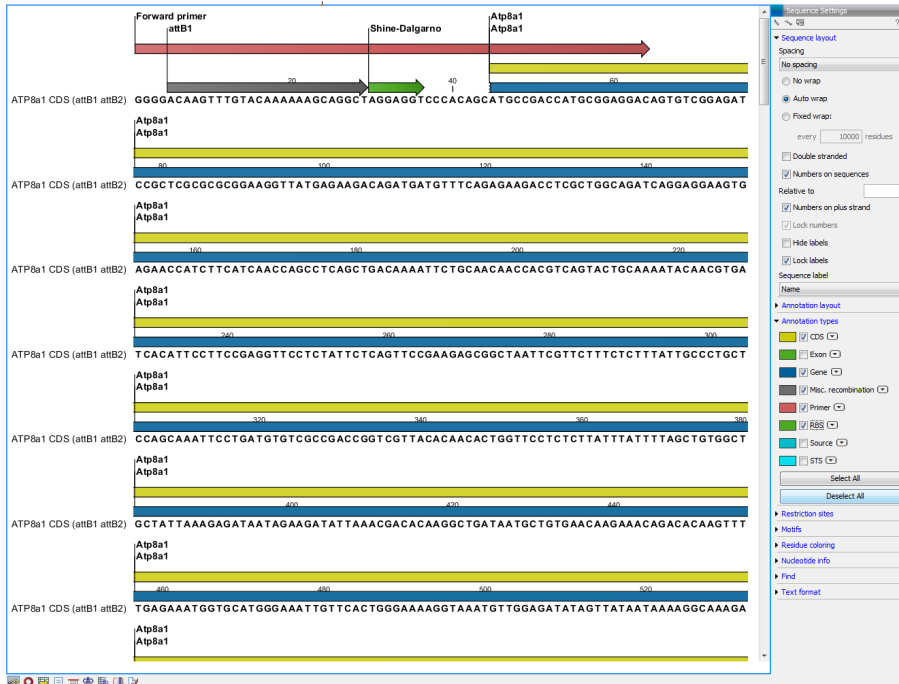


Figure 6: A view of the results: ATP8a1 CDS (attB1 attB2).

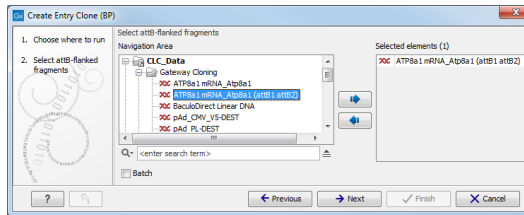


Figure 7: Select the sequence generated with the Add attB Sites tool.

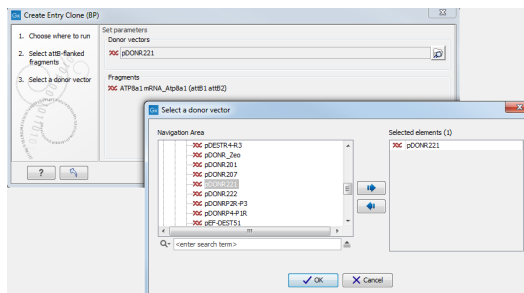


Figure 8: Select the donor vector, which in this case is pDONR221.

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**.

Open the output **pDONR221 (ATP8a1 CDS (attB1 attB2))** to have a look at it. To see a view like the one shown in figure 9:

- Go to circular view (🔄).
- 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.

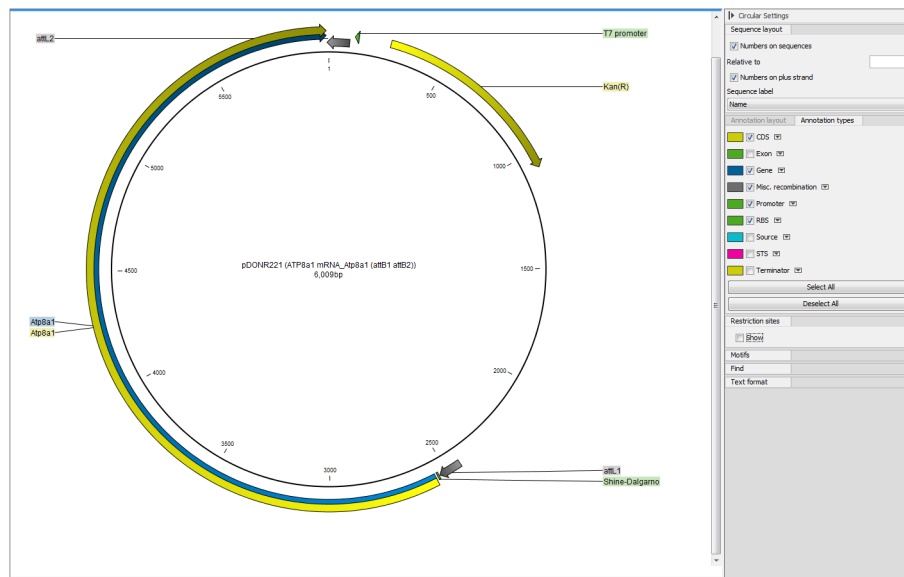


Figure 9: View of the entry clone, consisting of the donor vector, pDONR221, with the fragment of interest inserted by attB/attP recombination.

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 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:

Toolbox | Cloning and Restriction Sites (🔍) | 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 10).

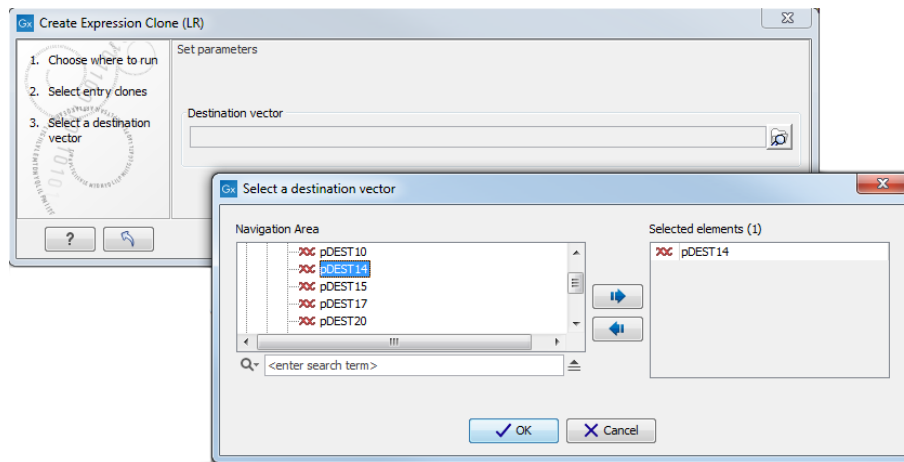


Figure 10: Select a destination vector.

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 11.

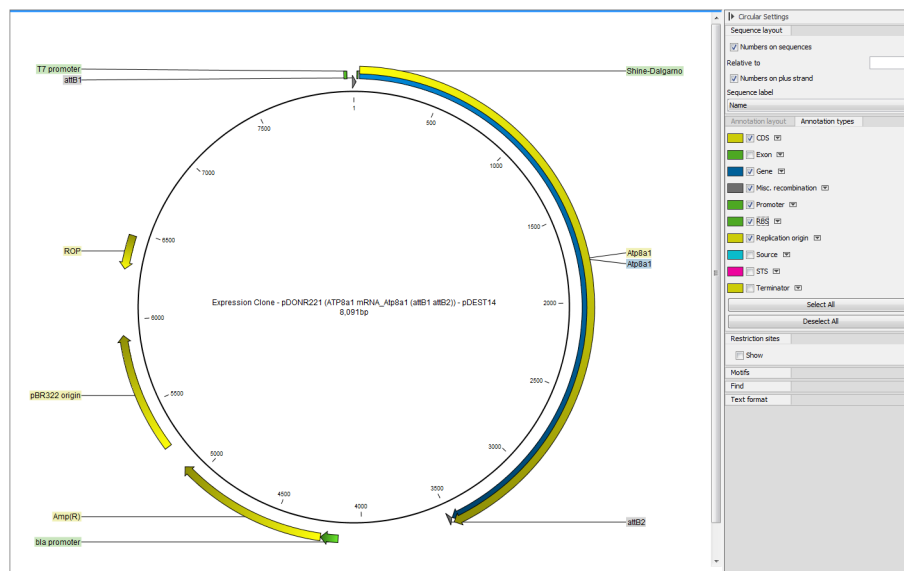


Figure 11: View the finished expression vector.

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

Short suggestion for a MultiSite Gateway Workflow using 2 fragments

The vectors mentioned in this section can be downloaded from Invitrogens web page:

<http://tools.invitrogen.com/downloads/Gatewayvectors.ma4>

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) + pDONR221-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