

Genotyping Analysis Module

USER GUIDE

for use with Diomni™ Design and Analysis (RUO) Software 3

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About the Genotyping Analysis Module

The Genotyping Analysis Module for Diomni™ Design and Analysis (RUO) Software 3 is used to detect single nucleotide polymorphism (SNP) variants of a target nucleic acid sequence.

For more information about genotyping analysis, see Chapter 5, “About genotyping analysis”.

IMPORTANT! If you omit wells or configure the analysis settings, click **Analyze** to reanalyze the data.

Genotyping analysis

Review results in the Amplification Plot (page 13)

Select the Genotyping Analysis Module (page 13)

Review results in the Allelic Discrimination Plot (page 13)

Omit outliers from genotyping analysis (page 16)

(Optional) Review dye signal profile in the Multicomponent Plot (page 17)

(Optional) Review signal profile in the Raw Data Plot (page 17)

Edit the genotyping analysis settings (page 17)



Set up a plate file

For detailed instructions about setting up a plate file, see the primary user guides for the software. See “Related documentation” on page 22.

Select a system template or existing plate file to set up a new plate file


A plate file contains the information that is necessary to perform an instrument run, including instrument setup, run method, plate setup, and analysis setting.

A system template is a non-editable plate file that is included with the software.

A new plate file must be created from a system template or a previously created plate file.

For detailed information about system templates and plate files, see the primary user guides for the software.

IMPORTANT! Select a system template or a plate file that corresponds to your instrument, block, and run mode. These properties are not editable after the plate file has been created.

1. Click  **Run templates**.
The **Plate Gallery** is displayed.
2. Click the tab at the top of the **Plate Gallery** that is associated with the type of plate file to be used to set up a new plate file.

Tab	Description
Recents tab	Contains plate files that were recently opened. Recently opened plate files from the System Templates tab and the My Plate Files tab do not populate this tab. Click a plate file to open it. The plate file can be edited, then saved, or saved as a new plate file.
My Plate Files tab	Contains plate files that were previously saved to the My Plate Files tab. Click a plate file to open it. The plate file can be edited, then saved, or saved as a new plate file.
System Templates tab	Contains system templates. System templates are non-editable plate files that are included with the software. Click a system template to automatically generate a new plate file that can be edited, then saved.

3. In the left pane, select the appropriate options to filter the system template and plate file lists.

- **Instrument**
- **Block**
- **Run Mode**
- **Analysis**

Note: Thermal protocol, plate setup, and post-run analysis options are independent of analysis module selection. Analysis module selection can be changed at any point during plate file setup or post-run analysis (see “Select the Genotyping Analysis Module” on page 13).

4. Open the plate file.

- Hover over the plate file, then click ... **(More Options)**.
The ... **(More Options)** menu for the plate file displays all of the options to open the plate file.
- Click the plate file.

The plate file opens in the **Run Method** tab.

Confirm or edit the run method

For most analysis, the default run method is appropriate. The following options are compatible:

- PCR
- 1-step RT-PCR
- 2-step RT-PCR
- In a plate file, in the **Run Method** tab, adjust the run method elements as needed.
For detailed instructions about editing the run method, see the primary user guides for the software.
- Click ... **(More Options)** ► **Filter Settings** to confirm or edit filter settings.

Confirm or edit the plate setup

For detailed instructions about plate setup, see the primary user guides for the software.

Add samples and assign to wells

For detailed instructions about plate setup, see the primary user guides for the software. See “Related documentation” on page 22.

1. In the **Plate Setup** tab, add samples and assign to wells using the following options.
 - Import a plate setup file.
 - Manually add samples to the **Samples** table.
To assign a sample from the **Samples** table to the well, select a well in the plate layout, then select the checkbox associated with the sample in the **Samples** table.
 - Manually add samples to wells in the plate layout.
The sample is added to the **Samples** table after it is added to the plate layout.

For the OpenArray™ Plate format, a sample layout can be imported. Samples cannot be added manually. The other formats allow a plate setup file to be imported, including targets and assays.

2. Confirm or edit sample information in the **Samples** table.

Column	Description
Name	Sample name
Color	Sample color
Type ^[1]	Genotyping analysis uses the following sample types. <ul style="list-style-type: none">• Unknown• Standard• Negative Control (NTC)• NEC• Positive Control• Positive 1/1• Positive 1/2• Positive 2/2

^[1] For more information, see “Sample types for genotyping analysis” on page 20.

3. Confirm or edit sample well assignments in the plate layout.

Add SNP assays and assign to wells


For detailed instructions about plate setup, see the primary user guides for the software.

SNP assays cannot be imported or assigned for the OpenArray™ Plate format.

1. In the upper-left corner of the **Plate Setup** tab, click **SNP**.
2. Add SNP assays and assign to wells using the following options:
 - Import an Assay Information File (AIF).
 - Import a plate setup file.
 - Manually add SNP assays to the **SNP Assays** table.
To assign an SNP assay from the **SNP Assays** table to the well, select a well in the plate layout, then select the checkbox associated with the SNP assay in the **SNP Assays** table.
 - Manually add SNP assays to wells in the plate layout.
The SNP assay is added to the **SNP Assays** table after it is added to the plate layout.
 - Import TaqMan™ assay plate and card files.
3. Confirm or edit target information in the **SNP Assays** table.

Column	Description
SNP Assay	SNP assay name
Color	SNP assay color
Allele 1 Reporter	Fluorescent reporter dye for allele 1 of the SNP assay
Allele 1 Quencher	Quencher for allele 1 of the SNP assay
Allele 2 Reporter	Fluorescent reporter dye for allele 2 of the SNP assay
Allele 2 Quencher	Quencher for allele 2 of the SNP assay
Task ^[1]	<p>The software automatically assigns a task to the target in a well based on the sample type in that well. The following tasks are used:</p> <ul style="list-style-type: none"> • Unknown • Standard • Negative Control (NTC) • NEC • Positive Control • Positive 1/1 • Positive 1/2 • Positive 2/2

^[1] For more information, see “Sample types for genotyping analysis” on page 20.

4. Click  (**Edit**) to open the **Edit SNP Assay** dialog box.
The **Edit SNP Assay** dialog box contains the information that was available in step 3.

5. Enter or edit the following information.
 - Assay ID
 - Gene symbol
 - Gene name
 - NCBI SNP reference
 - Context sequence
 - Allele 1 name
 - Allele 1 color
 - Allele 2 name
 - Allele 2 color
 - Comments
6. Confirm or edit SNP assay well assignments in the plate layout.

Edit reagent information

Reagents can only be edited for the TaqMan™ Array Card format and the OpenArray™ Plate format. Reagents cannot be added.

In the on-premise configuration, adding, editing, or deleting a reagent is a controlled function. The user role must have the permission of **Add/Edit/Delete Reagent**.

1. In the **Plate Setup** tab, in the **Targets/SNP Assays** table pane, click **Reagents**.
2. In the **Reagents** table, perform one of the following actions.
 - Click **+** (**Add**).
 - Click **...** (**More Options**) ▶ **Export Reagents** to export reagents.
 - Click **...** (**More Options**) ▶ **Import Reagents** to import reagents.
 - Click **...** (**More Options**) ▶ **Scan Reagents** to scan reagents.
3. If you are manually adding reagents or editing reagents, enter the following information in the table.

• Name	• Part Number
• Type	• Lot Number
• Barcode	• Expiration Date

Note: If the master mix that you enter is not compatible with the current run method, you have the option to apply the recommended run method for your master mix, instrument, block, and run mode.

4. If you are scanning the reagent barcode, in the **Scan Reagent** dialog box, select or deselect the **Enable automatic parsing** checkbox.

5. If you are scanning the reagent barcode, when the **Scan Reagent** dialog box is displayed, use a barcode scanner to scan the reagent label.

Note: If the master mix that you enter is not compatible with the current run method, you have the option to apply the recommended run method for your master mix, instrument, block, and run mode.

The fields in the **Scan Reagent** dialog box are populated.

6. In the **Scan Reagent** dialog box, click **Add**.
7. (Optional) Click **✕ (Remove)** in the row of a reagent to delete it from the table.

Select a passive reference

In the on-premise configuration, editing the passive reference is a controlled function. The user role must have the permission of **Edit Passive Reference**.


The passive reference is set for the plate. The default passive reference is ROX™ dye.

1. In the upper-left corner of the **Plate Setup** tab, select a passive reference from the dropdown list.
2. (Optional) Save the plate file or data file.

Review and save the plate file

In Thermo Fisher™ Connect Platform, instruments are connected by the InstrumentConnect application.

The QuantStudio™ 6 Pro Real-Time PCR Instrument and the QuantStudio™ 7 Pro Real-Time PCR Instrument can connect to the software.

1. In the **Run Summary** tab, review the run method selections, then edit if needed.
2. Review the plate setup, then edit if needed.
3. (Optional) Click the barcode field, then scan the plate barcode.
4. (Optional) Select **Add to My Plates**.
This option allows you to create new plate files using the current plate file as a template.
5. Select an instrument from the list.
If the instrument does not appear on the list, click  **System ▶ Instruments** to add a new instrument.
6. Save the plate file.

Start the run on an instrument. For more information about starting a run, see the documentation for the instrument.



Perform genotyping analysis

Review results in the Amplification Plot

For detailed instructions about reviewing results in the **Amplification Plot**, see the primary user guides for the software.

If no data are displayed in the **Quality Check** tab, or if reanalysis is required, click **Analyze**.

1. In the **Quality Check** tab, in the plot pane, select **Amplification Plot** from the dropdown list.
2. Review the amplification status for each well.
3. Review or edit threshold settings.
4. Review or edit baseline settings.

Select the Genotyping Analysis Module

1. In an open data file, click **Actions** ▶ **Analysis Modules**.
2. In the **Analysis Modules** window, select **Genotyping**, then click **Ok**.
The Genotyping Analysis Module opens.

Click **Analyze**, then review the results in the **Genotyping** tab.

Review results in the Allelic Discrimination Plot

For more information about the allelic discrimination plot, see “Allelic Discrimination Plot overview” on page 21.

If no data are displayed in the **Genotyping** tab, or if reanalysis is required, click **Analyze**.

1. In the **Genotyping** tab, in the upper-left corner of the **Allelic Discrimination Plot** pane, select **Scatter Plot** from the dropdown list (see Figure 1 on page 15).
The amplification plot can also be viewed in the **Genotyping** tab.
2. Select an assay from the **SNP Assay** list.
The Allelic Discrimination Plot is displayed for the selected assay.

3. Confirm that the data points for each genotype control cluster along the expected axis of the plot.

Table 1 Cluster assignments in an allelic discrimination plot

Content of samples	Location in AD plot
Allele 1 homozygote ^[1]	Lower right corner
Allele 2 homozygote ^[2]	Upper left corner
Alleles 1/2 heterozygote ^[3]	Approximately midway between Allele 1 and Allele 2 clusters
No-template control	Bottom left corner
Undetermined	Anywhere outside the regions described above
No amplification	With no-template control (NTC) cluster in the bottom left corner

^[1] Labeled with allele 1 fluorescent dye.

^[2] Labeled with allele 2 fluorescent dye.

^[3] Labeled with allele 1 and allele 2 fluorescent dyes.

4. Confirm that the cluster at the bottom-left corner of the plot contains only negative control wells. Samples can unexpectedly cluster with the negative controls for one of the following reasons.
 - Samples contain no DNA or a low concentration of DNA
 - Samples contain poor quality or degraded DNA
 - Samples contain PCR inhibitors
 - Samples are homozygous for a sequence deletion
5. Visually evaluate clusters for the three possible genotypes (see Figure 1 on page 15).

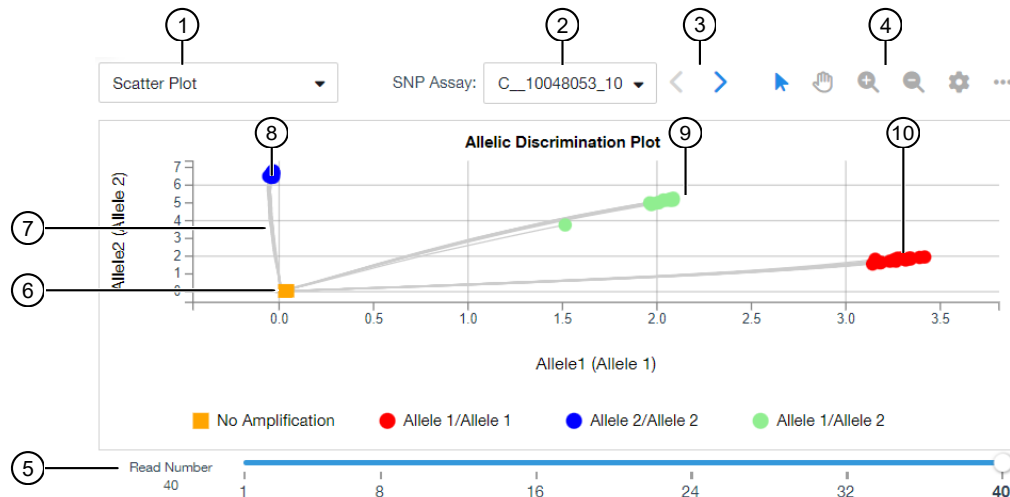


Figure 1 Allelic Discrimination Plot

- ① Plot type selection dropdown list
- ② Assay selection dropdown list
- ③ Assay selection navigation arrows
- ④ Plot toolbar
- ⑤ **Read Number** slider—For analysis that includes real-time data, the slider allows you to display the allelic discrimination data for all samples at each cycle during the PCR amplification.
- ⑥ NTC cluster
- ⑦ Real-time PCR amplification trails—For analysis that includes real-time data
- ⑧ Allele 2 homozygote cluster
- ⑨ Allele 1/2 heterozygote cluster
- ⑩ Allele 1 homozygote cluster

To confirm results, retest outliers and samples with no amplification (cluster with negative controls).

Perform a manual genotyping call

A manual call can be performed if the amplification is displayed far from the cluster in the allelic discrimination plot.

A manual call can be performed if a data point is outside of the algorithm for an automatic call. This produces an undetermined call even though there is valid amplification.

1. In the **Genotyping** tab, in the upper-left corner of the **Allelic Discrimination Plot** pane, select **Scatter Plot** from the dropdown list.
2. Select an assay from the **SNP Assay** list.
The Allelic Discrimination Plot is displayed for the selected assay.
3. In the table pane, click a well or multiple wells.

4. In the table pane, click ... **(More Options)**, then select the manual call option.

- **Allele 1 / Allele 1**
- **Allele 1 / Allele 2**
- **Allele 2 / Allele 2**
- **No amplification**
- **Undetermined**

5. To clear the manual calls, click ... **(More Options)** ▶ **Clear Manual Call**.

Genotyping calls

It is possible that genotyping calls are not generated due to no amplification even though there is amplification in the amplification plot.

It is possible that the reporter dye that is assigned one allele is brighter than the reporter dye that is assigned to the other allele.

To correct genotyping calls, change the assignment of the reporter dyes for each allele.

For example, if FAM™ dye was assigned to allele 1 and VIC™ dye was assigned to allele 2, change the assignment of the dyes. Assign VIC™ dye to allele 1 and assign FAM™ dye to allele 2.

The data must be reanalyzed after changing the assignment of the reporter dyes for each allele.

Omit outliers from genotyping analysis

Outlier wells have C_q values that differ significantly from the average for the associated replicate wells. To support C_q precision, consider omitting the outliers from analysis.

1. In the **Genotyping** tab, select an option to omit wells from analysis.

Option	Description
Omit wells in the Plate Layout	Select outlier wells, then click ... (More Options) ▶ Omit Wells .
Omit wells in the Well Table	Select Omit in the row of the outlier well.
Omit wells in the plot	Select the scatter plot from the dropdown list. Click and click and drag around the data to omit. The selected data are displayed in the Well Table and the Plate Layout . Omit the wells in the Well Table or the Plate Layout .

2. Click **Analyze** to reanalyze the data with any outliers removed.

(Optional) Review dye signal profile in the Multicomponent Plot

For more information about the **Multicomponent Plot**, see the primary user guides for the software.

If no data are displayed in the **Quality Check** tab, or if reanalysis is required, click **Analyze**.

1. In the **Quality Check** tab, in the plot pane, select **Multicomponent Plot** from the dropdown list.
2. Review the signal profiles for the passive reference dye, reporter dye, and negative control wells.
3. Review the plot to confirm that there are no irregularities in the dye signals.

(Optional) Review signal profile in the Raw Data Plot

For detailed instructions about reviewing results in the **Raw Data Plot**, see the primary user guides for the software.

If no data are displayed in the **Quality Check** tab, or if reanalysis is required, click **Analyze**.

1. In the **Quality Check** tab, in the plot pane, select **Raw Data Plot** from the dropdown list.
2. Click-drag the **Cycle Number** slider through all of the cycles, then confirm that each filter displays the characteristic signal increase.

Edit the genotyping analysis settings

1. In an open data file, click **Actions** ► **Genotyping Analysis Setting**.
2. In the **Genotyping Analysis Setting** dialog box, select an option in the **Analysis Data** section.
This setting determines the data that are used for genotyping analysis.

Analyze Data	Description
Post Read radio button	Only post-PCR read data is used to determine calls.
Pre Read & Post Read radio button ^[1]	The pre-PCR read is subtracted from the post-PCR read to determine calls.
Real Time Data radio button ^[2]	The normalized reporter data (Rn) from the selected cycle of the cycling stage is used to determine calls. Analysis is not restricted to the last cycle. Adjust the analysis cycle using the Cycle Slider in the Allelic Discrimination Plot pane (see Figure 1 on page 15).

^[1] The run method must include a pre-read stage.

^[2] Data collection must be on during the PCR stage.

3. Select or deselect the **Use Default** checkbox for each SNP assay.
The default settings are defined in the first row. Deselecting the **Use Default** checkbox enables the settings to be updated for each individual SNP assay.

If the default settings are updated, these settings are applied to each SNP assay.

4. Edit the number in the **Min Confidence %** field.

This is represented as a percentage. It is the confidence threshold. Edit the value to increase or decrease the stringency to make a genotyping call.

5. *(Real Time Data Only)* Enter values in the **Baseline Start** field and the **Baseline End** field.

The values are the first cycle and the last cycle that the software uses to calculate the baseline.

6. *(Real Time Data Only)* Enter a value in the **Cycle Number** field.

This value is the cycle number that is used to generate the normalized reporter data (Rn).

7. Click **Apply**.


The data are reanalyzed using the updated analysis settings.

Save images

Images of the allelic discrimination plot and the plate layout can be saved.

1. In the **Genotyping** tab, in the plot pane, select the plot type from the dropdown list.

- **Amplification plot**
- **Scatter plot**

Depending on the size of the monitor, you might need to click  (**List**) to view the dropdown list.

2. For the scatter plot, select the SNP assay.

- Select the SNP assay from the **SNP Assay** dropdown list.
- Use the arrows beside the **SNP Assay** dropdown list.

3. In the plot pane, click **⋮ (More Options) ▶ Save Image**.

4. In the plate layout pane, click **⋮ (More Options) ▶ Save Image**.

5. In the **Save Image** dialog box, enter or select the following information.

- Enter a file name in the **Filename** field.
- Select a file format radio button.
- Select a size from the **Size** dropdown list.

6. Click **Save Image**.

7. Navigate to the location to save the image, then click **Save**.

Export the results

In the on-premise configuration, the location to save the file is defined in the export settings. For users with the permission of **Edit Export Destination**, the location can be selected for a file download. For users without the permission of **Edit Export Destination**, the location cannot be selected.

In the Thermo Fisher™ Connect Platform, the results are exported to the downloads folder of the computer. The file name and location cannot be selected.

For more information about setting up the export location, see the primary user guides for the software.

1. In the table pane, click ... **(More Options)** ▶ **Export**.
In the Thermo Fisher™ Connect Platform, the file is downloaded.
2. *(Optional)* In the **Export CSV** dialog box, edit the file name in the **File Name** field.
The **File Name** field is populated with a default file name.
3. Click **Browse** to select a location to save the file.
The **Browse** button is not available in the on-premise configuration.
4. Click one of the following options.
 - Click **Download**, then select a location for the file download. This option is available for the on-premise configuration, for users who have the permission of **Edit Export Destination**.
 - Click **Save**. This option is available for the on-premise configuration, for users who do not have the permission of **Edit Export Destination**.
 - Click **Export**. This option is available in the desktop configuration.



About genotyping analysis

Overview of genotyping analysis

Genotyping analysis is used to detect single nucleotide polymorphism (SNP) variants of a target nucleic acid sequence.

Genotyping analysis uses preformulated TaqMan™ SNP Genotyping Assays that include the following components:

- Two sequence-specific primers for amplification of sequences containing the SNP of interest
- Two allele-specific TaqMan™ probes for Allele 1 and Allele 2

For genotyping analysis, the software performs the following tasks:

1. The software normalizes the fluorescence of the reporter dyes to the fluorescence of the passive reference dye in each well.
2. The software plots the normalized reporter dye signal of each sample well on an Allelic Discrimination Plot, which contrasts the reporter dye intensities of the allele-specific probes.
3. The software algorithmically clusters the sample data, and assigns a genotype call to the samples of each cluster according to its position on the plot.

Sample types for genotyping analysis

Genotyping analysis includes the following sample types.

Sample type (Type column in Samples table)	Sample description	Automatic target task assignment ^[1] (Task column in Targets table)
Unknown	Test sample	Unknown
Positive 1/1	Control sample that is homozygous for allele 1	Positive 1/1
Positive 1/2	Control sample that is heterozygous allele 1/allele 2	Positive 1/2
Positive 2/2	Control sample that is homozygous for allele 2	Positive 2/2
Negative Control	Water or buffer No amplification of the target should occur in NTC wells.	Negative control

^[1] The software automatically assigns a task to the target in a well based on the sample type in that well.

Allelic Discrimination Plot overview

The Allelic Discrimination Plot contrasts the R_n or the ΔR_n of the reporter dyes for the allele-specific probes of the SNP assay. It is an intermediary step in the software algorithm for genotyping calls.

A typical Allelic Discrimination Plot shows homozygote clusters, a heterozygote cluster, and the no-template controls. The points in each cluster are grouped closely together and each cluster is located well away from the other clusters. For expected cluster locations in the plot, see Figure 2 on page 21.

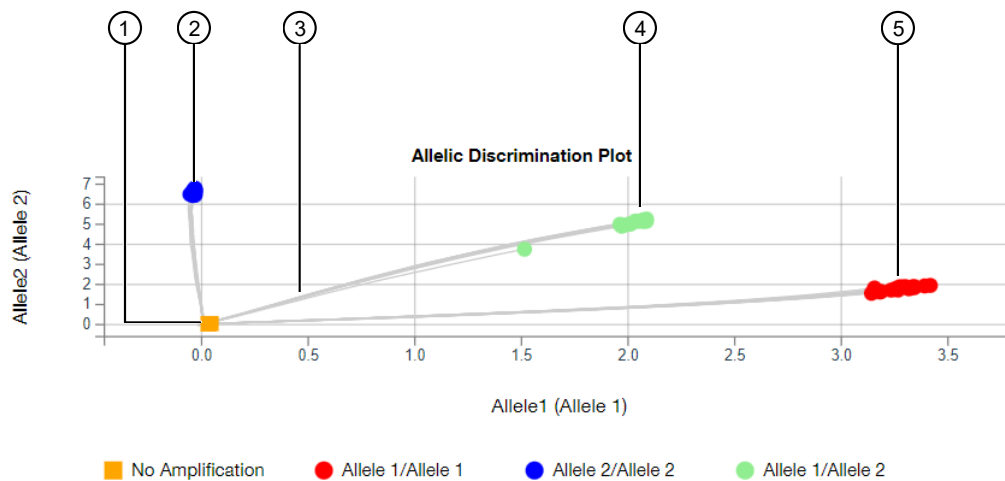


Figure 2 Example allelic discrimination plot

- ① No-template control cluster
- ② Allele 2 homozygote cluster
- ③ Real-time PCR amplification trails (for analysis that includes real-time data)
- ④ Allele 1/2 heterozygote cluster
- ⑤ Allele 1 homozygote cluster

Note:

- Undetermined samples may appear anywhere on the plot.
- No-amplification samples cluster with the no-template control samples.

Note: The clustering algorithm does not call genotypes if all the samples are one genotype (form one cluster).



Documentation and support

Related documentation

Document	Publication number
<i>Diomni™ Design and Analysis (RUO) Software 3 (Desktop) User Guide</i>	MAN0030162
<i>Diomni™ Design and Analysis (RUO) 3 User Guide (Thermo Fisher™ Connect Platform)</i>	MAN0030163
<i>Diomni™ Design and Analysis (RUO) Software 3 (On-Premise) User Guide</i>	MAN1000091

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