Azure Cielo 6 Real-Time PCR System compatibility with the GenDx KMRtype® and KMRtrack® Workflows

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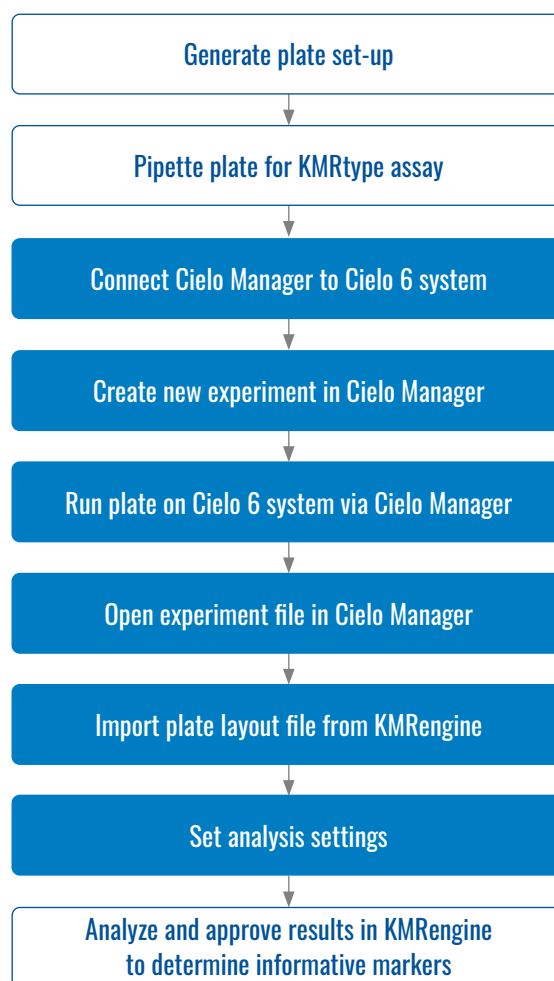[1]Azure Biosystems, 6747 Sierra Ct, Suite AB, Dublin, CA 94568, USA and
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Introduction

A bone marrow or hematopoietic stem cell transplantation is a treatment for leukemia that is intended to replace the patient's diseased bone marrow cells with healthy cells from a donor. After the transplantation, it is important to monitor the patient's chimeric state (now often a mixture of the donor and recipient genotypes) to ensure that the donor cells survive and stably outnumber the recipient's cells. An increase of the recipient genotype relative to the donor type indicates the patient's cells are multiplying faster than the transplanted healthy cells, crowding out the healthy blood cells and signaling a relapse of leukemia. Early detection of increasing mixed chimerism allows for earlier intervention and earlier intervention is associated with improved outcomes.

The KMRtype® and KMRtrack® kits from GenDx are designed for high-sensitivity monitoring of donor-recipient chimerism. The KMRtype kit is used to identify markers, called informative markers, that differentiate the donor cell genotype from the recipient cell genotype. KMRtype is a multiplex assay that requires a real-time PCR system such as the Cielo 6 that offers multiple fluorescent channels. KMR products are only IVD registered in EEA, Ukraine and Jordan.

Once informative markers are identified for a donor-recipient pair using KMRtype, two of those markers are used with the KMRtrack assay to monitor post-transplant samples for chimerism. KMRtrack is a singleplex assay that assesses the genotype of the patient's cells at the two informative markers, thereby determining whether and at what level recipient chimerism is present. KMRtrack can also reveal whether chimerism is increasing when used
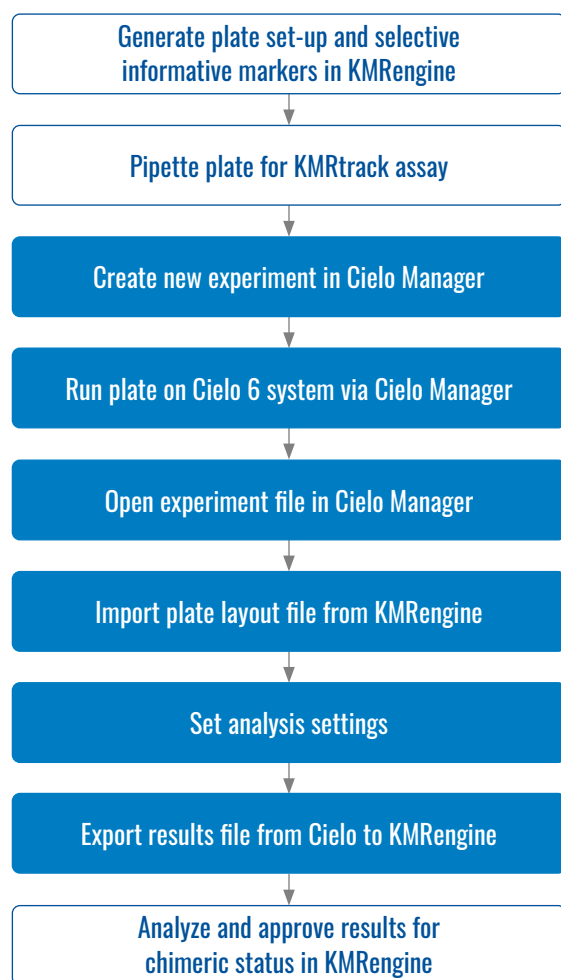

Figure 1. Overview of KMRtype workflow. The steps in blue are described in the application note. Steps in white are described in the KMRengine Instructions For Use (IFU) available from GenDx.

to assess the chimeric state of patient samples collected at successive time points after the transplantation.

The GenDx KMRtype and KMRtrack kits contain all assay components and reagents required to set up multi-well plate assays that are carried out using a real-time quantitative PCR instrument. A software package from GenDx, KMRengine, is used both to set up the plate layouts for the KMRtype and KMRtrack real-time PCR assays and to analyze the output of the quantitative real-time PCR assays. KMRengine identifies informative markers based on KMRtype results and quantifies the chimerism present based on KMRtrack results.

In this application note, we describe how to integrate the Cielo 6 Real-Time PCR system into the KMRtype and KMRtrack workflows (Figures 1 and 2) based on testing using a simulated chimerism case employing cell-line gDNA.

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│ Generate plate set-up and selective │
│  informative markers in KMRengine   │
└─────────────────────────────────────┘
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┌─────────────────────────────────────┐
│     Pipette plate for KMRtrack assay │
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┌─────────────────────────────────────┐
│   Create new experiment in Cielo Manager │
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                  ↓
┌─────────────────────────────────────┐
│ Run plate on Cielo 6 system via Cielo Manager │
└─────────────────────────────────────┘
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┌─────────────────────────────────────┐
│  Open experiment file in Cielo Manager │
└─────────────────────────────────────┘
                  ↓
┌─────────────────────────────────────┐
│  Import plate layout file from KMRengine │
└─────────────────────────────────────┘
                  ↓
┌─────────────────────────────────────┐
│        Set analysis settings        │
└─────────────────────────────────────┘
                  ↓
┌─────────────────────────────────────┐
│  Export results file from Cielo to KMRengine │
└─────────────────────────────────────┘
                  ↓
┌─────────────────────────────────────┐
│    Analyze and approve results for  │
│     chimeric status in KMRengine    │
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Figure 2. Overview of the KMRtrack workflow. The steps in blue are described in the application note. Steps in white are described in the KMRengine IFU available from GenDx.

Materials and methods
KMRtype Genotyping

Genomic cell-line DNA samples, representing a donor and recipient pair, were obtained from GenDx. Each sample was genotyped for 30 targets/markers using the first 10 mixes of the KMRtype kit. The plate layout and laboratory protocol were generated by KMRengine and the run was set up in a 96-well plate accordingly. Reactions were carried out in low-profile, opaque, white, 0.1 ml 96-well plates (Azure Biosystems AIQ322), sealed with Ultra Clear Optical Sealing Sheets (Azure Biosystems AIQ325). The plate layout file was exported toward the Azure Cielo Manager as a .TXT file. Duplicate runs were performed to ensure consistency of results.
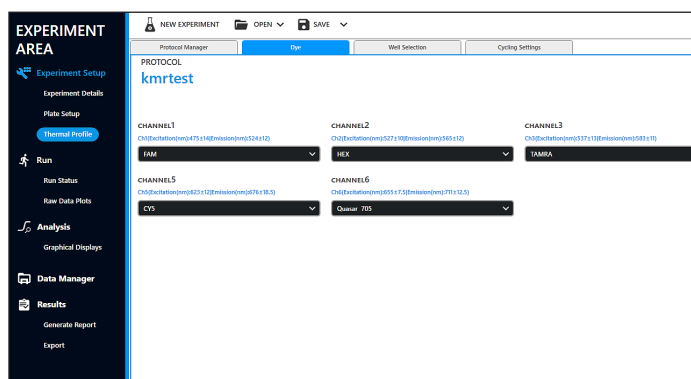
1. **Run plate on Cielo 6 system**

    Cielo Manager software was connected to Cielo 6 system via WI-FI, ethernet or USB. A new experiment was created on the Cielo Manager software. To create a new protocol, **Thermal Profile** was selected under **Experiment Setup** from the **EXPERIMENT AREA** menu to the left of the screen and the **Protocol Manager** tab was selected in the menu bar at the top of the screen. New Protocol was created and named. Dye tab was selected and dyes were assigned to each channel as recommended in the KMRengine IFU (Figure 3):

    Channel 1 FAM

    Channel 2 HEX

    Channel 4 ROX

Figure 3. Dye setup window. Note some channels are "on" but unused.

Next, the Well Selection tab was opened and it was confirmed that all channels were set to ON for all wells (Figure 4).

Then, the **Cycling Settings** tab was opened and the cycling parameters were set according to the instructions in the KMRengine IFU (Table 1, Figure 5).

The Auto Save function was turned on by toggling the circle to the bottom right of the PROTOCOL window. The file save location was selected by clicking "Browse" and the data file automatically saved as an .AZE file when the run was completed.

The assay was run by clicking the green **Run** button to the right of the **Cycling Settings** window.

Figure 4. Well selection window

Step		Temp	Time
Initial denaturation		95 °C	5 min
2-step cycling (40 cycles)	Denaturation	95 °C	15 sec
	Annealing/Elongation	62 °C	1 min

Table 1. Cycling Settings

Figure 5. Cycling settings window

2. Import plate set up from KMRengine

The data file (.AZE) was opened in Cielo Manager version 1.0.1.62 for data analysis. The Experiment Details screen was opened by selecting **Experiment Details** under **Experiment Setup** from the **EXPERIMENT AREA** menu on the left side of the screen. On the Experiment Details screen, **Import Plate Layout (.TXT)** was selected from the dropdown menu that appears when **OPEN** is selected in the menu bar at the top of the screen (see Figure 6). The file that had been exported from KMRengine was then located in the navigation window and selected for import.

The imported plate layout can be viewed by selecting **Plate Setup** from the menu on the left side of the screen (Figure 7).
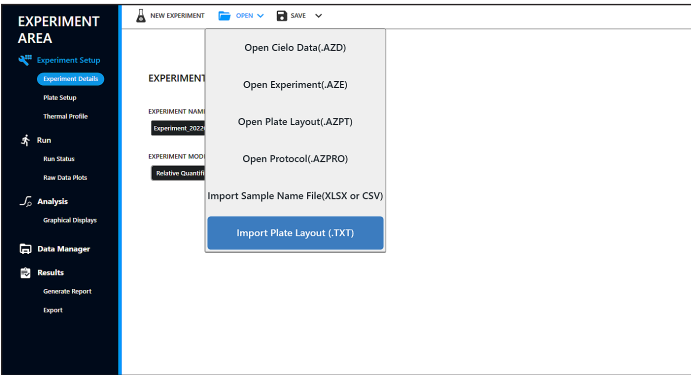

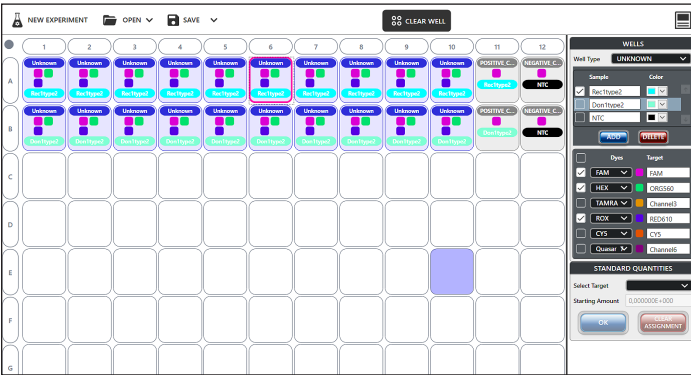Figure 6. Importing a plate layout file from KMRengine to Cielo Manager

Figure 7. View of imported plate layout for KMRtype run

3. Set analysis settings

Analysis settings were set by selecting Graphical Displays under **Analysis** in the **EXPERIMENT AREA** menu to the left of the screen and opening the **Amplification Curve** tab in the menu bar at the top of the screen. Clicking the SETTINGS icon at the top right of the screen opened a popup window to access analysis setting. Clicking the **General** tab of the popup window menu bar opened a screen in which the **Threshold Value** for each channel was set to 50000 and saved by clicking **OK** (Figure 8).

Next, on the **Baseline Settings** tab from the popup window menu, all channels were set to **Manual** baseline mode with a start value = 3 and an end value = 15 and saved by clicking **OK** (Figure 9).

Figure 8. Cq Algorithm Mode threshold settings

Figure 9. Baseline settings

4. Export results from the Cielo 6 to KMRengine

Data were then exported by selecting **Export** under Results in the **EXPERIMENT AREA** menu to the left of the screen. On the Export screen, the **TXT** tab was selected and the button **Export txt to GenDx KMRengine** was clicked (Figure 10).

The results were imported into KMRengine following the software instructions and analyzed. The informative markers found can be used to set up monitoring experiments for the genotyped recipient-donor pair.
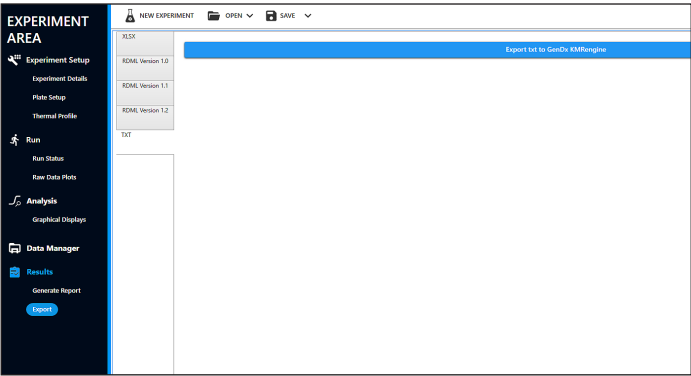

Figure 10. Export results to GenDx KMRengine

KMRtrack Chimerism Analysis

The KMRtrack kit was used with samples provided by GenDx. One time point (emulated post-transplant recipient sample) was analyzed for two different artificial chimerism samples with two markers each. Selected markers were previously found to differentiate the "recipient" and "donor" genotypes. The samples and markers used are shown in Table 2.

The experimental design and plate layout were created in KMRengine and the plate layout exported as a .TXT file. Reactions were carried out in low-profile, opaque, white, 0.1 ml 96-well plates (Azure Biosystems AIQ322), sealed with Ultra Clear Optical Sealing Sheets (Azure Biosystems AIQ325).

Sample	Marker 1	Marker 2
Rec13AzureS	KMR016	KMR035
Rec14S	KMR038	KMR057

Table 2. Samples and markers used in KMRtrack chimerism analysis

1. **Run plate on Cielo 6 system**

   Cielo Manager software was connected to Cielo 6 system via WI-FI, ethernet or USB. A new experiment was created on the Cielo Manager software. To create a new protocol, **Thermal Profile** was selected under **Experiment Setup** from the **EXPERIMENT AREA** menu to the left of the screen and the **Protocol Manager** tab was selected in the menu bar at the top of the screen. New Protocol was created and named. Dye tab was selected and dyes were assigned to each channel as recommended in the KMRengine **IFU** (Figure 11):

   Channel 1 FAM

   Next, the **Well Selection** tab was opened and it was confirmed that all channels were set to **ON** for all wells (Figure 12).

   Subsequently, the **Cycling Settings** tab was opened and the cycling parameters were set according to the KMRengine IFU (Table 3, Figure 13).

   The Auto Save function was turned on by toggling the circle to the bottom right of the PROTOCOL window. The file save location was selected by clicking "Browse" and the data file automatically saved as an .AZE file when the run was completed.
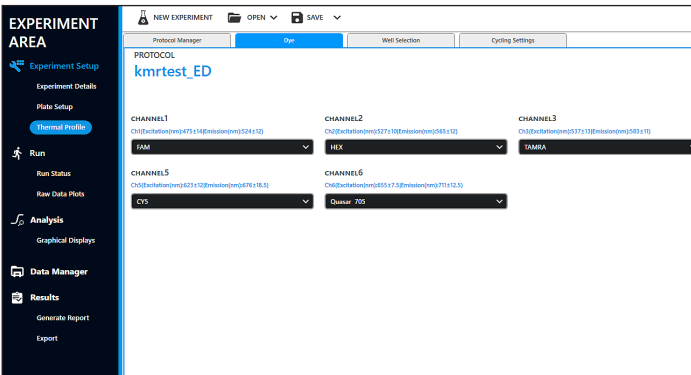
The assay was run by clicking the green **Run** button to the right of the **Cycling Settings** window.

2. **Import plate set up from KMRengine**

   The data file (.AZE) was opened in Cielo Manager version 1.0.1.62 for data analysis. The Experiment Details screen was opened by selecting **Experiment Details** under **Experiment Setup** from the **EXPERIMENT AREA** menu on the left side of the screen. On the Experiment Details screen, **Import Plate Layout (.TXT)** was selected from the dropdown menu that appears when **OPEN** is selected in the menu bar at the top of the screen (see Figure 14). The file that had been exported from KMRengine was then located in the computer's file system and selected for import.

Step		Temp	Time
Initial denaturation		95 °C	5 min
2-step cycling (40 cycles)	Denaturation	95 °C	15 sec
	Annealing/Elongation	62 °C	1 min

Table 3. Cycling Settings

Figure 11. Dye setup window. Note some channels are "on" but unused.

Figure 13. Cycling settings window

Figure 12. Well selection window. Note some channels are "on" but unused.
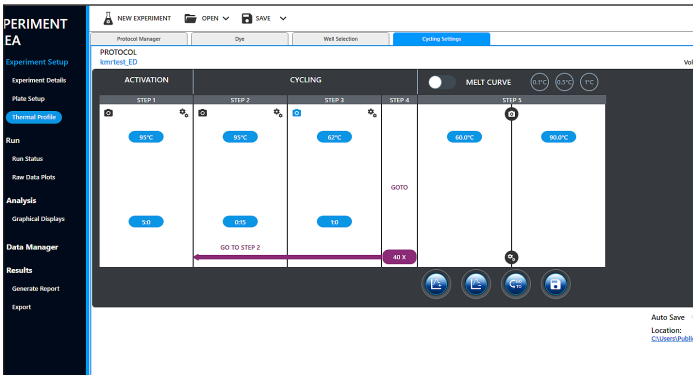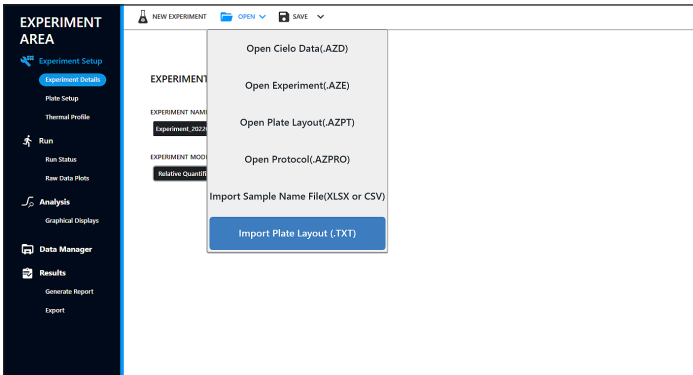

Figure 14. Importing a plate layout file from KMRengine to Cielo Manager

The imported plate layout was viewed by selecting **Plate Setup** from the menu on the left side of the screen (see Figure 15).

3. **Set analysis settings**

Analysis settings were set by selecting Graphical Displays under **Analysis** in the **EXPERIMENT AREA** menu to the left of the screen and opening the Amplification Curve tab in the menu bar at the top of the screen. Clicking the SETTINGS icon at the top right of the screen opened a popup window to access analysis setting. Clicking the **General** tab of the popup window menu bar opened a screen in which the **Threshold Value** for each channel was set to 50000 and saved by clicking **OK** (Figure 16).
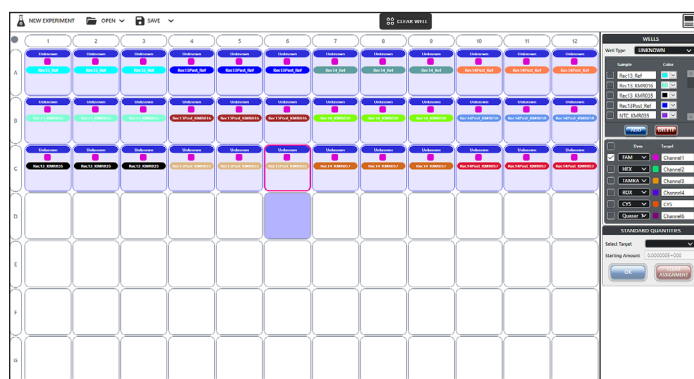
Next, on the **Baseline Settings** tab from the top menu of the popup window, all channels were set to **Manual** baseline mode with a start value = 3 and an end value = 15 and saved by clicking **OK** (Figure 17).

4. **Export results from the Cielo 6 to KMRengine**

Data were then exported by selecting **Export** under **Results** in the **EXPERIMENT AREA** menu to the left of the screen. On the Export screen, the **TXT** tab was selected and the button **Export txt to GenDx KMRengine** was clicked (Figure 18).

The results were imported into KMRengine following the software instructions. KMRengine software used the ΔΔCq method with the experimental Cq values to calculate the percentage of recipient chimerism.

Figure 15. View of imported plate layout for KMRtrack run
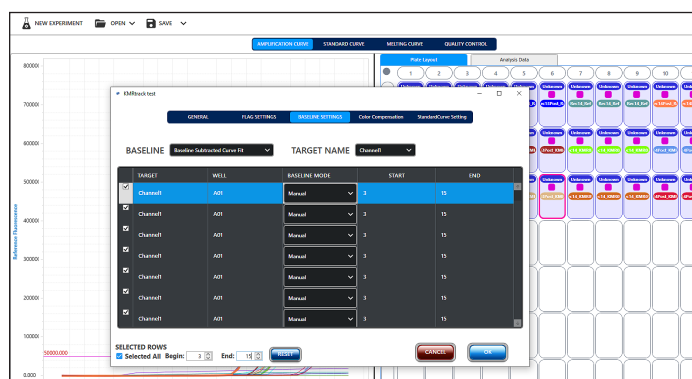

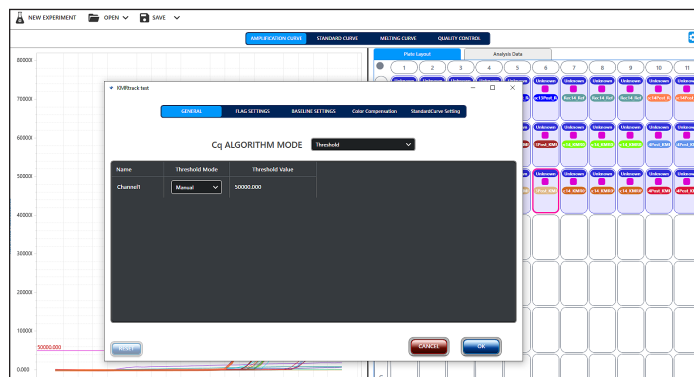Figure 17. Baseline settings

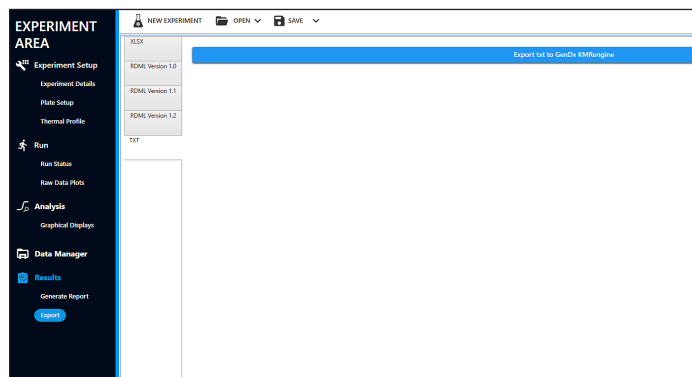Figure 16. Cq Algorithm Mode threshold settings

Figure 18. Export results to GenDx KMRengine

Results

KMRtype

Genomic cell-line DNA samples, representing a donor and recipient pair, were genotyped for 30 markers each on the Cielo 6 using the KMRtype kit (Figure 19). Two runs using the same samples were performed, by different operators on different days. The resulting data were successfully transferred to KMRengine for analysis (Figure 20). The genotyping results found in both runs were 100% concordant with each other as well as with the expected results for these known samples (Figure 21).

KMRtrack

One time point (emulating a post-transplant recipient sample) was analyzed for two different artificial chimerism samples. Each "patient" sample was analyzed using two informative markers demonstrated by the KMRtype kit to differentiate the DNA of "donor" from the "recipient". Experiments for both "patients" were combined on one plate.
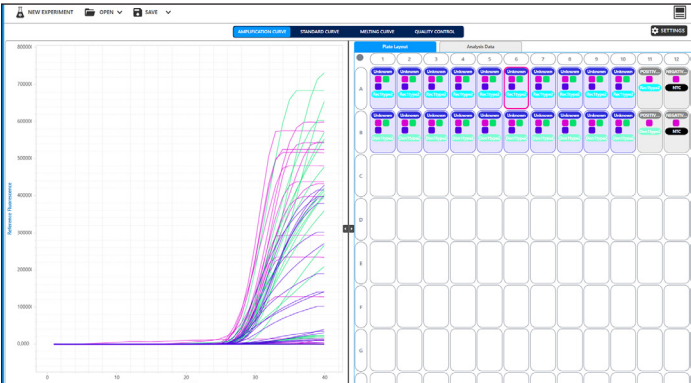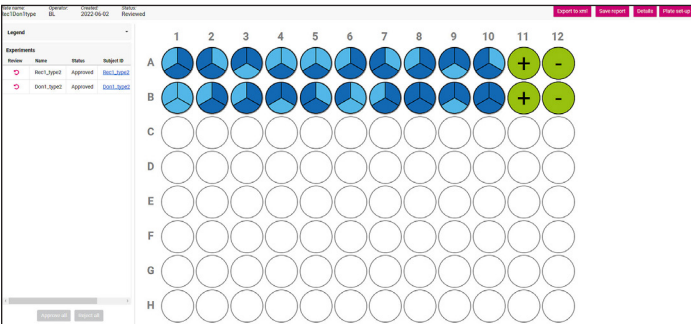

Figure 19. KMRtype output

Figure 20. KMRengine import and analysis of the 2nd run of the typing plates

Upon importing the data into KMRengine, all negative controls passed and the variation between triplicate reactions was below 2% C.V., yielding a valid result for all wells included (Figure 22).

The ΔΔCq method was then used to assess chimerism in each sample, using the generated Cqs for each marker normalized to the reference of its sample and then to a 100% recipient reference sample. If chimerism would increase over time, this would indicate an increase in the relative amount of recipient cells and a high potential for relapse. The current two experiments show only a starting point of such graphs. An example of the output of the monitoring assay is shown in Figure 23.

Channel	Type mix	well	Pre-type	Run 1	Run 2	well	Pre-type	Run 1	Run 2	Informative for
FAM		A1	+	28	29	B1	-	40	40	Recipient
HEX	Mix 01	A1	+	30	30	B1	-	40	40	Recipient
ROX		A1	-	40	40	B1	-	40	40	-
FAM		A2	-	40	40	B2	+	27	27	Donor
HEX	Mix 02	A2	+	29	29	B2	+	29	29	-
ROX		A2	+	29	29	B2	-	40	40	Recipient
FAM		A3	+	28	29	B3	+	28	29	-
HEX	Mix 03	A3	-	40	40	B3	+	31	31	Donor
ROX		A3	+	29	29	B3	-	40	40	Recipient
FAM		A4	-	40	40	B4	+	29	29	Donor
HEX	Mix 04	A4	+	27	27	B4	-	40	40	Recipient
ROX		A4	-	40	40	B4	+	29	30	Donor
FAM		A5	-	40	40	B5	-	40	40	-
HEX	Mix 05	A5	+	29	29	B5	+	28	28	-
ROX		A5	-	40	40	B5	+	29	29	Donor
FAM		A6	+	26	27	B6	-	40	40	Recipient
HEX	Mix 06	A6	+	28	29	B6	-	40	40	Recipient
ROX		A6	-	40	40	B6	+	29	29	Donor
FAM		A7	-	40	40	B7	+	29	29	Donor
HEX	Mix 07	A7	+	28	29	B7	+	29	29	-
ROX		A7	+	31	32	B7	-	40	40	Recipient
FAM		A8	+	27	27	B8	+	27	27	-
HEX	Mix 08	A8	+	28	28	B8	+	29	30	-
ROX		A8	-	40	40	B8	+	29	30	Donor
FAM		A9	+	28	28	B9	+	28	28	-
HEX	Mix 09	A9	-	40	40	B9	-	40	40	-
ROX		A9	+	29	29	B9	+	30	30	-
FAM		A10	-	40	40	B10	+	28	29	Donor
HEX	Mix 10	A10	+	29	29	B10	+	28	28	-
ROX		A10	+	28	28	B10	+	29	29	-
FAM	Ref901	A11	+	27	28	B11	+	28	28	
FAM		A12	-	40	40	B12	-	40	40	

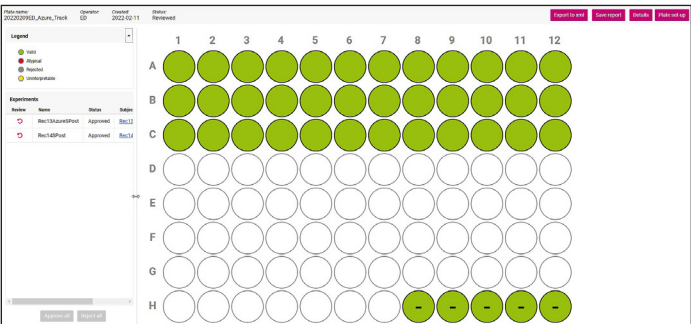**Figure 21. Resulting Cq values compared between runs and to pre-type generated by GenDx**

Figure 22: KMRengine import and analysis of the monitoring plate.

The results found for each marker using the Cielo 6 as well as the previous reference from GenDx are shown in Table 4.
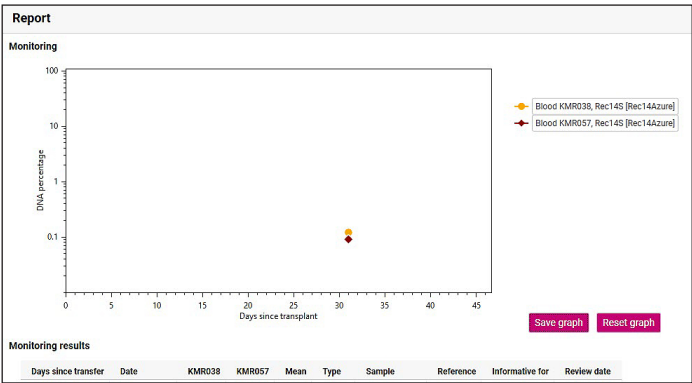

Figure 23. Monitoring output from the analysis of sample Rec14S at one time point

Marker	ΔΔCq	% Recipient Chimerism	% Recipient Chimerism (reference GenDx)
(Emulated) recipient 13			
KMR016	3.57	8.39	7.6
KMR035	4.73	3.77	3.12
Mean % reported	—	**6.08**	**5.36**
(Emulated) recipient 14			
KMR038	9.66	0.12	0.08
KMR057	10.16	0.09	0.09
Mean % reported	—	**0.11**	**0.08**

Table 4. Chimerism analysis results

Conclusion

The Cielo 6 Real-Time PCR system is easily integrated into the KMRtype and KMRtrack workflows and delivers reliable results in both genotyping and monitoring. It is straightforward to import plate layout files from KMRengine to the Cielo 6, and to export qPCR data from the Cielo 6 to KMRengine for analysis. In the experiments carried out in this note using known test samples provided by GenDx, all qPCR results obtained using the Cielo 6 agreed with the expected results, confirming the Azure Cielo 6 is compatible with both the KMRtype genotyping and the KMRtrack monitoring kits.