protocol v1.07

Digital PCR – QIAcuity One 5plex

Generalities

A good introduction on dPCR: https://www.qiagen.com/de/knowledge-and-support/knowledge-hub/bench-guide/pcr/digital-pcr/what-is-digital-pcr

QIAcuity systems are designed to determine absolute amounts of target DNA or RNA in a sample by using a digital PCR approach. The possible applications are:

- absolute quantification
- mutation detection
- genome editing analysis
- copy number variation (CNV)
- gene expression analysis

For each one of these applications, the QIAcuity software has a dedicated secondary analysis module

The strengths of dPCR are:

- No need for standard curves since absolute quantification
- high tolerance to inhibitors (since end-point detection)
- high precision (10% changes detected)
- High sensitivity (single events can be spotted)
- No PCR efficiency issues (high reproducibility)
- Assays and probes are generally directly transposable from qPCR to dPCR

Putative weaknesses of dPCR are:

- Shallower dynamic range than qPCR (about 4 logs vs 7 logs), hence dilutions may be needed to fall in the sweet range
- No melting curve and impossible to collect amplicons after the run, so may need optimization in case the specificity of the assay is unclear.
- No real time, only end point, so may be difficult to identify false positive partitions.

Some characteristics:

- End-point PCR
- PCR reaction in many individual partitions
- Amplicon sizes with an ideal range of 60bp-150bp
- Target molecule presence is detected by measuring the fluorescence by:
    - sequence specific DNA probes
    - or intercalating dyes

- Total number of filled partitions is identified by a passive fluorescent dye, present in the reaction mix
- Nanoplate-based system:
  - 8.5k partitions (24 or 96 wells)
  - 26k partitions (8 or 24 wells) → maximal sensitivity and dynamic range
- Workflow
  - Partitioning
  - Thermocycling
  - Imaging

Nanoplates

- Touch nanoplates only on the sides
- Place the nanoplate into the nanoplate tray to avoid damaging the optical surface and to keep it clean
- Do not centrifugate the nanoplate as this will lead to pre-priming and insufficient filling of the wells
- Do not vortex nanoplates as this leads to insufficient filling of the wells
- Transport the nanoplate horizontally
- You can reimage the plate e.g., if it was overexposed

User Instructions

For more details, please refer to the QIAcuity User Manual.

dPCR booking

Training is required before using the QIAcuity One 5plex. Once you've completed the training, you can book the instrument through PPMS: https://sv-ppms.epfl.ch/.

Depending on the method used, please book the instrument for 1.5 to 3 hours per plate.

The booking does not include plastics and reagents. You can either bring your own nanoplates or purchase them from us (they are available next to the instrument). Please ensure you indicate the type and number of GECF nanoplates on the log sheet located next to the instrument.

Pricing

EPFL internal prices are available here:

https://www.epfl.ch/research/facilities/gene-expression-core-facility/page-154614-en-html/

Setting up the QIAcuity software

Login

For the QIAcuity PC

- user name -> QIAcuity PC
- password -> admin

For the QIAcuity software

- user name: admin
- password: admin

- EPFL users can directly connect through the browser: https://128.178.194.12:8687/Plates

Creating a new experiment

- Refresh the web page to make sure the connection is still functional.
- Click new plate
- Enter plate name (please name it as such: PI_User_Date_XXX) and plate type
- Editing the plate barcode is optional

dPCR parameters

- Priming section:
  - Select master mix
- Cycling section:
  - Select cycling parameters (refer to master mix/probes user guide)
  - Enter each step and temperature
  - Steps can be grouped, edited or deleted
- Gradient cycling:
  - This gradient temperature functionality allows cycling at different temperatures across the columns of a QIAcuity Nanoplate. This feature, only available on 8.5K 96-well nanoplates, supports assay development by enabling easy identification of the optimal temperature in a single-plate run. This can be specially useful for complicated samples such as environmental samples of FFPE samples.
- Imaging section:
  - unselect channels which are not required
    - Green channel: FAM, SYBR Green, EvaGreen
    - Yellow channel: VIC, HEX
    - Orange channel: TAMRA
    - Red channel: ROX
    - Crimson channel: Cy5
  - exposure time and gain settings are applied automatically
  - Depending on the assay different exposure time and gain settings might be required. Avoid oversaturation (warning appears during result analysis). The plate can be imaged again after the run with different settings if needed.

Reaction mixes

- Fill reaction mix name
- Fill target name
- Fill detection channel
- Click create

Samples & controls

- Click new sample
- Fill name and amount
- Click create
- For a bulk of samples set the amount to the sample amount needed: 3,4 etc
- For controls and NT controls: enter name and add control

Plate layout

- Select reaction mix
- Right click and select sample
- Assign
- Assign empty wells as blank
- Or for many samples import plate layout (we can provide templates)
  You can also import whole plate data from a CSV file to define the plate layout (Please refer to the QIAcuity User Manual for more details).

Starting a run

Template DNA fragmentation

Not required for DNA <20kb (e.g., cDNA, FFPE DNA or circulating DNA, cfDNA, or genomes < 20 kb)

- Restriction digestion is needed for DNA of average lengths of ≥ 20 kb (e.g., genomic DNA purified via spin columns with silica membrane)
- Selected restriction enzymes can be added into the reaction mix. EcoRI-HF, PvuII, XbaI (6-cutters), AluI, CviQI, HaeIII (4-cutters) digest template DNA in 10 minutes at room temperature in the QIAcuity PCR Master Mix.
  Ensure that the enzyme does not cut in the amplicon.

Reaction set up using the QIAcuity EG PCR kit (Ref: 250111, 250112, 250113)

- The final volume depends on the nanoplate type used
- Never vortex or centrifuge the QIAcuity nanoplate

Component	**Nanoplate 8.5K (24-well, 96-well)**	**Nanoplate 26K (8-well, 24-well)**	**Final concentration**
MasterMix	x µl	x µl	1x
10x primer mix *or* 10x primer-probe mix	x µl	x µl	

Restriction Enzyme (optional)	*Up to 1 µl*	*Up to 1 µl*	*0.025-0.25 U/µl*
RNase-free water	variable	variable	
DNA (add in plate)	variable	variable	Must be in linear range of the plate (dilutions can be valuable).
Total reaction volume	**12 µl**	**40 µl**	

1. Prepare the reaction mix corresponding to the MasterMix you are using (without the DNA template). Restriction Enzyme is optional.
2. Vortex the mix
3. Dispense appropriate volumes of reaction mix to tubes or the wells of a PCR plate
4. Add DNA template
5. Mix and vortex the tubes or PCR plate
6. *Optional: incubate 10 minutes in case enzyme was added for template fragmentation*
7. Handle the nanoplate carefully (never touch the bottom) and place it on a clean Nanoplate tray.
8. Transfer the content of each well to the corresponding well of the nanoplate
9. Make sure that no air bubbles are introduced into the wells of the dPCR plate during pipetting (bubble on top is less of a problem).
10. Seal the nanoplate using the QIAcuity Nanoplate Seal
11. Use the nanoplate roller 3-4 times vertically and horizontally
12. Remove the transparent foil
13. Use the nanoplate roller 3-4 times vertically and horizontally and then roll over the plate frame

Loading the plate into the QIAcuity instrument

1. Transport of the Nanoplate horizontally and touch only the sides
    a. *It is recommended to cover the plate with the top seal within 30 minutes after pipetting to prevent subsequent filling issues. Run should be started directly (maximum 2 hours)*
2. Press the button on the instrument or the Eject Tray on the touchscreen
3. Place the plate in the tray
    a. Correct orientation: facing the barcode toward the instrument and the QIAGEN lettering toward you
4. The barcode of the loaded plate is scanned
5. Press the icon on the touch screen, and select an existing experiment defined in experiment
6. If the barcode does not match any plate in the software suite, use the control software to define the experiment. Enter Plate name etc.

The following actions can be performed when your run is finished

a. To reimage the plate with different settings, click +, then select Imaging
b. To rerun the plate with additional cycles, click +, then select Cycling + Imaging

Important: After a run is finished, remove the plate from the instrument before adding a new imaging or cycling + imaging step.

Checking quality of your images

1. Click on the plate and select analysis
2. If the imagine quality is good there are no warnings (proceed with result analysis)
3. If exposure times or gains were too high, the imagine symbol will be highlighted in yellow. In this case you can reimage the plate with e.g., 30% lower exposure time:
    a. Press the back button
    b. Select dPCR parameters
    c. Click on + and select + imaging
    d. Reduced exposure duration (eg.700ms to 490ms)

Plate storage: Store plates at room temperature or in the fridge (without light). Plates can be reimagined still a few days later

Results analysis

Select the selection you would like to use

- Absolute Quantification
- Mutation detection
- Genome Editing
- Copy Number Variation
- Gene expression

e.g., for Absolute Quantification

- Select the wells
- Select Target or detection channel
- Click show results
    - In list: the concentration, confidence interval, as well as positive and negative partitions are shown (Export as csv)
    - For an overview select heatmap: heatmap of the target channel will be shown
    - To change threshold values, select on heatmap, 1D scatterplot or 2D Scatterplot: Move the threshold or type in the value. Then click recalculate

How to handle errors

- Red color
- Click tools
- Select the red marked box
- Reboot instrument
- If a plate was aborted during a run the plate can be run again
- Save the plate and load it again in the instrument
- Edit and run to start

Which metrics to QC in priority?

- The percent of positive wells should be ideally between 20% and 90%. A range between 10% and 99% is still considered OK. Below 10% positive partitions, if the separation between negative and positive partitions is clear, data are still perfectly analyzable, but the confidence interval is higher due to subsampling effect.
- The separation between positive and negative populations should be clear. If you have a "rain" pattern, your assay needs to be optimized.

Diverse notes

Mastermixes

Only Qiagen mastermixes can be used with the QIAcuity instrument. The list is available here:

https://www.qiagen.com/us/product-categories/discovery-and-translational-research/pcr-qpcr-dpcr/dpcr-assays-kits-and-instruments/dpcr-kits

Instructions for QIAcuity Software Suite Installation

The QIAcuity software suite can be installed on a PC not connected to the instrument to perform analyses. It is important that the version installed on your PC matches the version on the instrument's PC (as of April 2025: version 3.1). Once the software is installed and opened, it will automatically connect to the homepage for identification. By default, the username is "admin" and the password is also "admin".

Exporting and Importing Data

To perform analyses, you need to export the raw data from the software suite connected to the instrument and then import them into your own system for further analysis. Note that the exported zip file cannot be unzipped (it is password protected by Qiagen themselves), it can only be imported in the Qiagen Suite for further processing. Once imported, the raw images can be accessed through the "analysis" tab, "Check source images" button on the top right on the interface.

Multiplexing

- Multiplexing different targets (with different dyes) is more robust for dPCR than qPCR, because of the end point nature of dPCR.

- Multiplexing 3 targets is routine, 4 still generally easy, and 5 is less common and should be optimized.

- The orange channel on the QIAcuity is the only one requiring fluorescence compensation with other channels. Therefore only use it if doing 5-plex.

Gene expression analysis

- The advantage of dPCR-based gene expression analysis is the possibility to detect down to 10% changes. For that it is recommended to use a one-step mastermix, that will partition the RNA and perform the cDNA/PCR directly inside the partitions. See above for the list of mastermixes.

Troubleshooting

If your problem is not listed below, refer to the QIAcuity user manually, or contact us.

- While designing the plate before the run, when trying to save you may get this error “The plate could not be saved due to a problem with server connection». → Solution: Refresh the webpage (plate design will be lost unfortunately).

Changelog

- v1.01: initial release
- v1.02: many small changes
- v1.03: remove the plate from the instrument after the run
- v1.04 (07.04.2025): Mentioned procedure to export raw data, and to access the raw images upon re-import. QIAcuity Software Suite Installation, pricing, booking and gradient cycling details have been added. Information regarding the CSV file used to define the plate layout has been added. Clarified how long to book per run.
- v1.05 (16.04.2025): Mentioned the different types of nanoplates available.
- v1.06 (20.04.2025): Mentioned we can provide templates for plate layout import. Clarified that gradient are only available on 8.5K 96-well nanoplates. Clarified the strengths of dPCR.
- v1.07 (30.06.2025): Refresh the web page before starting a design to make sure the connection is still functional. Added a section about multiplexing. Indicated that 26k is best when sensitivity and dynamic range are important. Weaknesses list added. Indicated important metrics QC. Added a section about gene expression analysis. Indicated that only Qiagen mastermixes can be used.