MAQ Control for
WG MAQ and ROI MAQ

Version 1.1

For CNV analysis

IFU511
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Chapter 1: Introduction

1.1. Multiplex Amplicon Quantification

Multiplex Amplicon Quantification (MAQ) is a straightforward method for the detection and analysis of copy number variations (CNVs).

It consists of the simultaneous PCR amplification of several fluorescently labeled target and reference amplicons. After fragment analysis, comparison of the relative intensities of the target amplicons in the test individual and a control individual result in the copy number of those target amplicons.

1.2. MAQ Control

MAQ Control concept allows the design and development of a ROI MAQ assay for a specific genomic region of interest, or selection of a predesigned Whole Genome (WG) MAQ assay via the supplementary data tracks within the Nexus Copy Number software (BioDiscovery).

1.2.1. ROI MAQ Design

ROI MAQ assays can be designed free of charge using MAQ Design service at www.multiplicom.com. One human genomic region of interest can be specified (at least 30 kb, positions based on NCBI built 37.1). After confirmation, primer pairs for maximally five target amplicons are returned by email. The amplicons are evenly spread throughout the region and are designed to work in conjunction with a universal set of six reference amplicons, present in MAQ Control (Cat. no. YM-0092.100).

1.2.2 WG MAQ selection

WG MAQ assay is a predesigned, whole genome CNV detection tool that is integrated as supplementary data tracks within the Nexus Copy Number software (BioDiscovery). This novel integrated approach enables combined visualization of the WG MAQ assays and copy number events previously detected with NGS and microarray. This enables the customized selection of a WG MAQ for flexible and fast CNV confirmation based on the proven MAQ concept. After selections, primer pairs for maximally five target amplicons are returned. The amplicons are designed to work in conjunction with a universal set of six reference amplicons, present in MAQ Control (Cat. no. YM-0092.100).

1.2.3 MAQ Control assay development

To be able to perform a customized MAQ Control experiment, the user needs to order primer pairs and determine the optimal primer concentrations in the final MAQ reaction through a straightforward and robust optimisation protocol.

1.2.4 MAQ Control analysis

After the optimisation is performed, the customized MAQ Control assay can be used to determine the copy number of the genomic region of interest in a high-throughput manner.

1.3. More information

More details (e.g. publications and application notes) about ROI MAQ, WG MAQ and MAQ Control can be found at www.agilent.com.

Chapter 2: Before you begin

This chapter describes the main prerequisites to perform a MAQ Control analysis, as detailed in chapter 3 of this user guide. Before starting, make sure that all the requirements listed in this section are met. This section is divided into the following:

Section 1.1: Physical installations
Section 1.2: Materials

2.1. Physical installations

Always take precautions to prevent contamination of DNA samples. To minimize the risk of contamination, make a clear-cut distinction between pre-PCR and post-PCR laboratories and research workflows:

- Pre-PCR work:
  - Genomic DNA concentration measurements
  - PCR reaction setup
- Post-PCR work:
  - PCR cycling
  - Fragment analysis reaction setup
  - Fragment analysis on (automated) fragment analyser
- Use physically separated pre-PCR and post-PCR laboratories
- Use distinct sets of pipettes and consumables for pre-PCR and post-PCR research workflows

2.2. Materials

2.2.1. DNA samples

The quality and quantity of the DNA samples are critical to the success of this procedure. DNA degradation and PCR inhibitors will be reflected in the quality of the MAQ analysis.

The protocol requires a total of 20-50 ng input DNA, meeting the following requirements:

- $OD_{260/280}$ ratio ≥ 1.7
- Concentration ≥ 10 ng/µl in H_2O
- No DNA degradation visible on agarose gel

To prevent degradation of DNA samples, always follow these guidelines:

- Prevent freeze-thaw cycles
- Long-term storage: -20 °C

- Short-term storage: 4 °C
- Keep your DNA stocks at a high concentration (> 50 ng/µl) in TE or TE^{-4}
- Make dilutions for your MAQ analysis in sterile distilled H_2O

2.2.2. Primers for reference amplicons

The designed primers for the target amplicons in the genomic region of interest need to be ordered from an oligo-provider, desalted and without modifications. We recommend storing the primers at a concentration of 100 µM at -20 °C in several aliquots to prevent numerous freeze-thaw cycles, as these influence the quality negatively.

2.2.3. MAQ Control

Each MAQ Control kit contains reagents for the analysis of at least 100 DNA samples:

Vial name	Storage	Components
PCR mix	-20 °C	PCR buffer, dNTPs, primer pairs of the six control amplicons
Taq polymerase	-20 °C	Taq DNA polymerase

2.2.4. Materials required but not provided

Materials	Requirements
Reference samples	See 1.2.1 DNA samples. These samples contain a normal copy number of the region under investigation.
Positive control	See 1.2.1 DNA samples. This sample contains a known deviation from the normal copy number of the region under investigation. This sample is not absolutely required.
HiDi™ formamide	ABI cat. no. 4311320
GeneScan®500Liz™ size standard	ABI cat. no. 4322682
GeneScan®500Rox™ size standard	ABI cat. no. 401734

Chapter 3: Procedure

A MAQ Control analysis comprises of 3 steps:

Section 3.1: PCR reaction
Section 3.2: Fragment analysis
Section 3.3: Evaluation of the results

! Attention: DNA contamination

To reduce risk of DNA contamination, perform all steps of section 3.1 until 3.1.4 (the PCR cycling) in a pre-PCR laboratory that is physically separated from the post-PCR laboratory and use dedicated pipettes that are exclusively used in pre-PCR research workflows.

3.1. PCR reaction

PCR setup should be performed on ice to obtain the best results. Start the PCR reaction as soon as possible after addition of the Taq DNA polymerase.

3.1.1. DNA sample dilution

1. Measure DNA concentration if unknown.
2. Dilute the DNA to 10-20 ng/µl, using sterile distilled water.
3. Include a negative control (only H_2O) and if possible a positive control. Always include reference individuals (≥2) with known normal copy number.

3.1.2. Preparation of primer mix

1. After receiving the primers of your designed multiplex, adjust them to a final concentration of 100 µM.
2. If this is the first step in the optimisation process of this MAQ Control, prepare two primer pools:
    a. one containing 150 nM of each F primer
    b. one containing 900 nM of each R primer

   Hereto, add for the F primer pool 1.5 µl of each primer (100 µM) in a total volume of 1,000 µl, and for the R primer pool 4.5 µl of each primer (100 µM) in a total volume of 500 µl.
3. If it is not the first step, prepare the two primer pools according to previously optimised primer concentrations.
4. Vortex briefly.

! It's recommended to store the primers at -20 °C and to make aliquots of the primer stock solution to avoid frequent freeze-thaw cycles.

*3.1.3. Preparation of **master reaction mix***

1. Remove the PCR mix from the -20 °C freezer and allow complete thawing on ice.
2. Vortex thoroughly and centrifuge the vials at 12,000 x g for 10 s before use.
3. On ice, prepare a **master reaction mix** for the number of samples and controls to be run (minimum 8) + 1. In one microcentrifuge tube, add the following reagents, in the order indicated:

3	µl/rxn	MAQ Control PCR mix
1	µl/rxn	F primer pool
1	µl/rxn	R primer pool
0.075	µl/rxn	Taq DNA polymerase
5.075	µl/rxn	final volume

4. Vortex briefly and centrifuge the vials at 12,000 x g for 10 s.

! Incomplete mixing of the viscous glycerol enzyme containing solution with the PCR mix is a major source of errors.

3.1.4. PCR reaction setup

1. Label 0.2 ml tubes or a 96-well PCR plate.
2. Prepare the PCR mix in the tubes/wells by adding the following reagents, in the order indicated:

2-10 µl	gDNA with a total DNA amount between 20-50 ng
0-8 µl	Sterile distilled water
5 µl	Master reaction mix
*15 µl	Final volume

! * Add the 5 µl **Master reaction mix** to the DNA samples using separate pipette tips and mix by pipetting up and down.

3. Briefly vortex and centrifuge the vials or PCR plate until all liquid is at the bottom.

3.1.5. PCR cycling

1. In a post-PCR room, place all vials or PCR plate firmly in the thermal cycler block and start the PCR cycle program.

98°C – 10 min		
95°C – 45 s	}	x25
60°C – 45 s		
68°C – 2 min		
72°C – 10 min		

! Use a ramp rate below 2.5 °C/s. A higher ramp rate can influence the amplification reproducibility.

2. After completion of the PCR, the MAQ PCR product may be stored at 4 °C for up to 5 days before analysis by capillary electrophoresis.

! As the fluorescent labels used are light-sensitive, the PCR products should be stored in a dark box or wrapped in aluminium foil.

3.2. Fragment analysis

3.2.1. Preparation of size standard mix

1. Prepare a size standard mix for the number of samples to be analysed. In a micro-centrifuge tube, add per sample the following reagents:

10 µl/rxn HiDi-Formamide (not supplied; see section 2.2.3)
0.3 µl/rxn GS500 size standard (not supplied; see section 2.2.3)

2. Briefly vortex and centrifuge the vial until all liquid is at the bottom.

3.2.2. Preparation of sample for fragment analysis

1. For each sample, dispense 10 µl of the size standard mix into a well of a 96-well plate compatible with the fragment analyser used for MAQ analysis (not supplied).
2. Add 2 µl of the MAQ PCR product to a well containing the size standard mix.
3. Seal the plate with an aluminium sealing foil (not supplied).
4. Denature the samples at 95 °C for 3 min and put on ice immediately.
5. Centrifuge the plate at 1,000 x g for 10 s to remove any bubbles in the wells and load onto the fragment analyser

3.2.3. Fragment analysis

Module for the ABI3730XL sequence analyser:

Run Module Settings

Name	Value	Range
Oven_Temperature	66	18...70 DegC
Buffer_Temperature	35	30...35 DegC
PreRun_Voltage	15.0	0...15 kV
PreRun_Time	180	1...1800 sec
Injection_Voltage	2.0	0...15 kV
Injection_Time	10	1...90 sec
First_ReadOut_Time	200	100...16000 ms
Second_ReadOut_Time	200	100...16000 ms
Run_Voltage	15.0	0...15 kV
Voltage_Number_Of_Steps	10	0...100 Steps
Voltage_Step_Interval	20	0...180 secs
Voltage_Tolerance	0.6	0...6.0 kV
Current_Stability	30.0	0...2000 uA
Ramp_Delay	1	1...1800 sec
Data_Delay	120	1...1800 sec
Run_Time	1200	300...14000 sec

! Please inquire to obtain the fragment analysis settings for other ABI sequencers. Some parameters can change when using other types of fragment analyser.

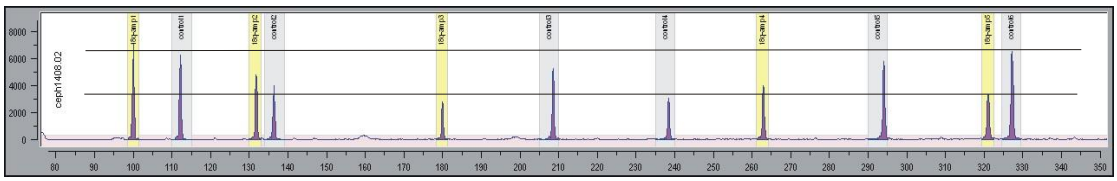
3.3. Evaluation of the results

3.3.1. Data analysis

For data analysis, we recommend the MAQ-S software package (downloadable from www.agilent.com). A separate User Guide for MAQ-S is available.

3.3.2. Optimization of peak heights

Evaluate the peak heights of your target amplicons in the obtained chromatograms. Try to get roughly equal peak heights for all amplicons by adapting the primer concentration in the primer mix and repeating the multiplex PCR and GS (from 3.1.2)



Ideally, peak heights of all amplicons should be more or less equal in height. If this is not the case after the first round of optimization, reiterate the process (3.1.2.) starting from the individual primer sets as follows:

If a target amplicon peak in the chromatogram is **low** in comparison with the reference amplicon peaks, increase the concentration in the primer mix with steps of 150 nM for the F primers and 900 nM for the R primers with a maximal concentration of the primer in the primer mix of 2 µM for the R primer.

If a target amplicon peak in the chromatogram is **high** in comparison with the reference amplicon peaks, decrease the concentration in the master primer mix with steps of 50 nM for the F primers and 300 nM for the R primers.

Repeat step 3.1.2 to 3.3.2 using the adapted primer concentrations until the peak heights of the target amplicons are roughly equal to those of the reference amplicons.

From this moment on, customized MAQ Control assay is ready to be used for the copy number analysis of your genomic region of interest in your samples, using the same protocol as used during the optimisation.

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