iQ-Design Assays

User Guide

Tests for the real-time PCR detection of microorganisms in food and environmental samples

BIO-RAD

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Section 1
Introduction

iQ-Design Assays are specially designed for the rapid detection and characterization of microorganisms in food, water, and environmental samples. Using real-time polymerase chain reaction (PCR), specific DNA sequences for the target microorganisms are amplified and detected simultaneously by means of fluorescent probes. The intended users of this kit are trained laboratory personnel who are performing tests to detect microorganisms in food and environmental samples. The use of this test allows results to be obtained within a few hours following enrichment of a sample.

Section 2
The iQ-Design Assay Technology

The iQ-Design Assay products are available as individual primer-probe mix reagents that are wet-lab validated for use with Bio-Rad's iQ Multiplex Powermix amplification reagent. The iQ-Design Assays are designed and optimized for the iQ-Check DNA extraction workflows and compatible with Bio-Rad's CFX96 Touch Deep Well Real-Time PCR Detection System.

The ready-to-use assay contains oligonucleotides (primers and probes) specific for target microorganisms. PCR is a powerful technique used to generate many copies of target DNA, called amplicons. During this chain reaction, several cycles of heating and cooling lead to exponential accumulation of these amplicons, making it eventually possible to detect the target DNA. Each cycle starts with the denaturation of the double-stranded DNA at a high temperature. When the temperature decreases, the primers bind to the target region and are extended by the DNA polymerase. Two amplicons are thus generated from each initial target DNA fragment.

In real-time PCR, specific probes bind to target DNA and are lysed by the DNA polymerase during extension. These probes are linked both to a fluorophore and a quencher, which absorbs the light emitted by the fluorophore. Upon lysis of the probe, the fluorophore and quencher are separated so that fluorescence is released. In the absence of target DNA, no fluorescence will be detected. If target DNA is present in the sample, as the number of amplicons increases with each round of amplification, fluorescence intensity also increases. At each PCR cycle, the optical module, or detector, measures this fluorescence. The software associated with the instrument plots fluorescence intensity against the number of cycles. This method allows a simple determination of the presence or absence of the target microorganism in a sample.

Section 3
Kit Components

The iQ-Design Assay is a ready-to-use primer-probe mix optimized for use with iQ Multiplex Powermix. Each kit comes with 0.8 or 1.6 ml of assay mix, sufficient for 100 or 200 reactions, respectively.

Section 4
Shelf Life and Storage

iQ-Design Assays are shipped at room temperature. Upon receipt, store product at 4°C (up to 12 months). For long-term storage up to the expiration date indicated on the vial, store at –20°C.

If storing the vial at –20°C, prevent oligonucleotide degradation by aliquoting the total volume into smaller volumes to minimize the number of freeze-thaw cycles.

Section 5
Materials Required but Not Supplied

Equipment

- 10, 20, 200, and 1,000 µl micropipets
- Bio-Rad CFX96 Touch Deep Well Real-Time PCR Detection System (catalog #3600037)

Note: We recommend using an uninterrupted power supply (UPS) with the thermal cycler.

Supplies

- iQ Multiplex Powermix (catalog #17006573, 200 reactions; 17007081, 100 reactions)
- PCR plates, tubes, sealing tape, and caps
- Sterile filter tips adaptable to 10, 20, 200, and 1,000 µl micropipets
- Tips for Combitip pipets or equivalent repeat pipettors; sterile, individually packaged
- 2 and 5 ml sterile test tubes
- Powder-free gloves
- Distilled sterile water
- Bleach, 5%
- Cleaning agent such as DNA AWAY or RNase AWAY

Section 6
Safety Precautions and Recommendations for Best Results

- This test must be performed by trained personnel

- Samples and enrichment cultures must be handled as potentially infectious material and discarded according to local rules and regulations
- All potentially infectious material should be autoclaved before disposal
- The quality of results depends on strict compliance with Good Laboratory Practices (for example, the EN ISO 7218 standard), especially concerning PCR
    - Never circulate laboratory equipment (pipets, tubes, etc.) from one workstation to another
    - Always use a positive control and a negative control for each series of amplification reactions
    - Do not use reagents after their expiration date
    - Vortex reagents from the kit before using to ensure homogeneity
    - Periodically verify the accuracy and precision of pipets, as well as correct functioning of the instruments
    - Change gloves often, especially if you suspect they are contaminated
    - Clean workspaces periodically with 5% bleach and other decontaminating agents such as DNA AWAY
    - Use powder-free gloves and avoid fingerprints and writing on tube caps. Both will interfere with data acquisition
- It is strongly advised to follow the general requirements described in the standard EN ISO 22174:2005 (Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food pathogens — General requirements and definitions)
- iQ-Design Assays
    - All substances or mixtures in the test kit are classified products, according to the Globally Harmonized System (GHS). Contact with acids may cause release of toxic gases. No special precautions are necessary if used correctly. If the product is inhaled, supply fresh air and consult a doctor in case of complaints. After eye contact with the product, rinse opened eye for several minutes under running water. If the products are swallowed, induce vomiting and call for medical help
- CFX96 Touch Deep Well Real-Time PCR Detection System
    - Improper use of the CFX96 Touch Deep Well Real-Time PCR Detection System may cause personal injury or damage to the instrument. Some components may pose a risk of personal injury due to excessive heat if improperly handled. For safe use, the CFX96 Touch Deep Well Real-Time PCR Detection System must be operated only by qualified laboratory personnel who have been appropriately trained. Servicing of instrument must be performed only by Bio-Rad Field Service Engineers
- For iQ-Design Assays that do not include an internal control, we recommend performance verification with your matrix prior to analysis. Certain matrices may contain PCR inhibitors that could impede the amplification reaction. To ensure that amplification proceeds properly, run two tests in parallel:
    - Perform a 1:10 dilution of the target DNA used for the positive control in distilled sterile water
    - Perform a 1:10 dilution of the same target DNA in a DNA extract prepared after sample enrichment

Important: The matrix sample used for this enrichment must be free of naturally occurring target DNA. Inhibition of the reaction will show a shift of the amplification curve toward a higher quantification cycle (Cq refers to the cycle at which the amplification curve crosses the threshold) compared to the Cq value of the first test (partial inhibition), or as signal extinction (complete inhibition). For samples showing inhibition (partial or complete), repeat the test by performing a 1:10 dilution of DNA extract in sterile distilled water.

Section 7
Protocol

A. Sample Enrichment and DNA Extraction

The iQ-Design Assays are designed for use with DNA extraction samples obtained from the Standard or Easy DNA extraction protocols. Refer to the iQ-Design Assay Technical Report for your specific assay to find further details on the sample enrichment and DNA extraction protocol.

B. Real-Time PCR

Instrument and software setup

For instrument setup, follow instructions in the CFX96 Touch Deep Well Instruction Manual (Bulletin #10021337). For software setup instructions, see Chapter 3, Performing Runs.

PCR mix preparation

1. Prepare PCR mix containing the amplification solution (iQ Multiplex Powermix) and the fluorescent probe mix (iQ-Design Assay Primer-Probe Mix). Volume of PCR mix needed depends on the number of samples and controls to be analyzed. At least one positive and one negative control must be included in each PCR run. Use the pipetting table in the Appendix to find the correct volumes to use for each reagent.

2. After preparation, the PCR mix (iQ Multiplex Powermix + iQ-Design Assay Primer-Probe Mix) should be used immediately or within 1 hr, stored at 2–8°C.

3. Pipet 20 µl of PCR mix into each well according to your plate setup.

4. Add 5 µl of DNA extract and negative or positive control. Do not vortex the sample before pipetting. Hermetically seal the wells of the plate or strips. It is important to avoid bubbles at the bottom of the wells by pipetting carefully. As an optional step to eliminate any bubbles, centrifuge the sealed PCR plate or the PCR strips (quick spin).

5. Place the plate or strips in the thermal cycler. Be sure to place the plate with the A1 well at the upper left corner. Close the reaction module.

Run PCR

1. Open CFX Manager Software to set up the PCR conditions.

2. On the main window toolbar, click **User-defined Run Setup**.

3. Create a new protocol: on the Protocol tab, click **Create New** to open the Protocol Editor to create a new protocol (see Figure 1).

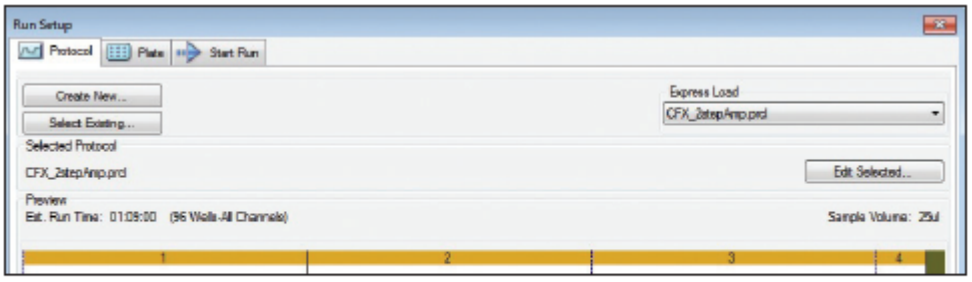


Fig. 1. Protocol tab in the Run Setup window. Load an existing protocol or create a new protocol for a run.

4. Select any step in either the graphical or text display — the step becomes highlighted in blue (see Figure 2). Click the temperature or dwell time to directly edit the value. Refer to the iQ-Design Assay Technical Report for the thermal cycling conditions specific to your assay. Click **Insert Step** or **Delete Step** to add or remove a temperature step from the protocol.

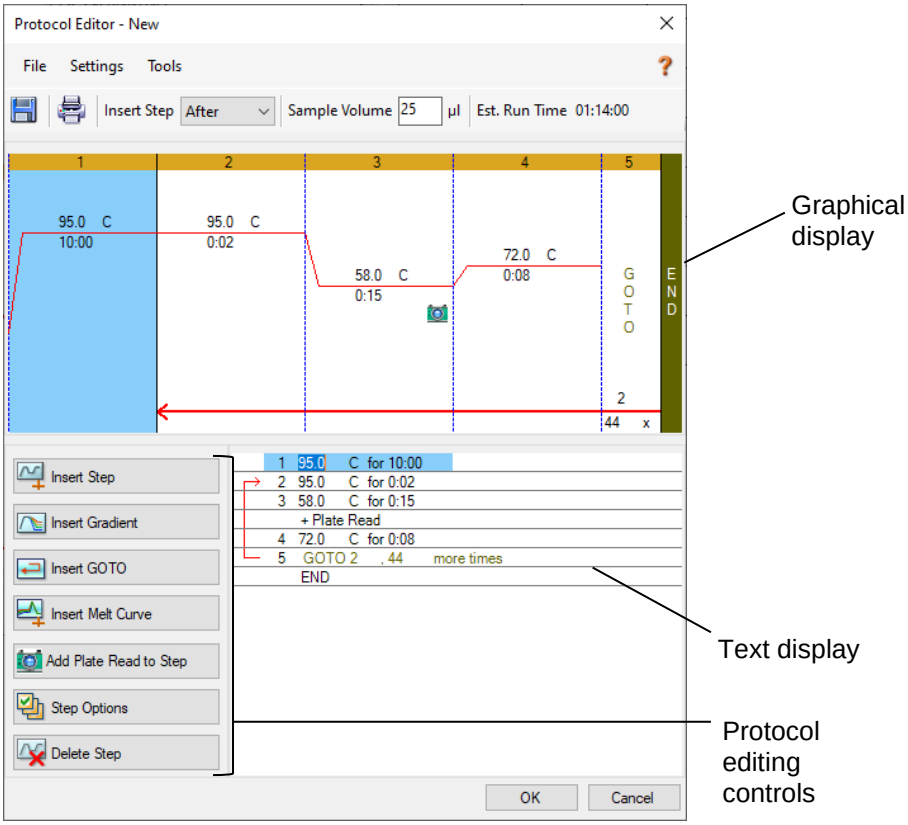


Fig. 2. Protocol Editor. The protocol editing controls are on the left. Both the graphical and text displays can be edited. Step 1 is highlighted and 95°C is selected for editing in the text display.

5. Click **Add Plate Read to Step** to designate when the fluorescence data will be acquired during the protocol. Add the Plate Read step after Step 3.

6. Click the number of repeats of a GOTO step to change the number of cycles in the protocol. Click the GOTO step number to change the steps included in the GOTO loop following the number of cycles in Figure 3.

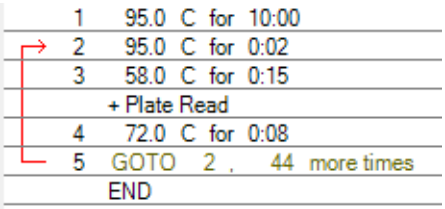


Fig. 3. An example of an iQ-Design Assay thermal cycling condition.

7. Once the protocol is set up, click **OK** and save the current protocol with the name specific to your iQ-Design Assay.

8. On the Plate Tab, select **Create New** to open the **Plate Editor** window (see Figure 4).

9. Click **Select Fluorophores** and indicate the fluorophores of interest (see Figure 5). Refer to the iQ-Design Assay Technical Report for the fluorophore type specific to your assay.

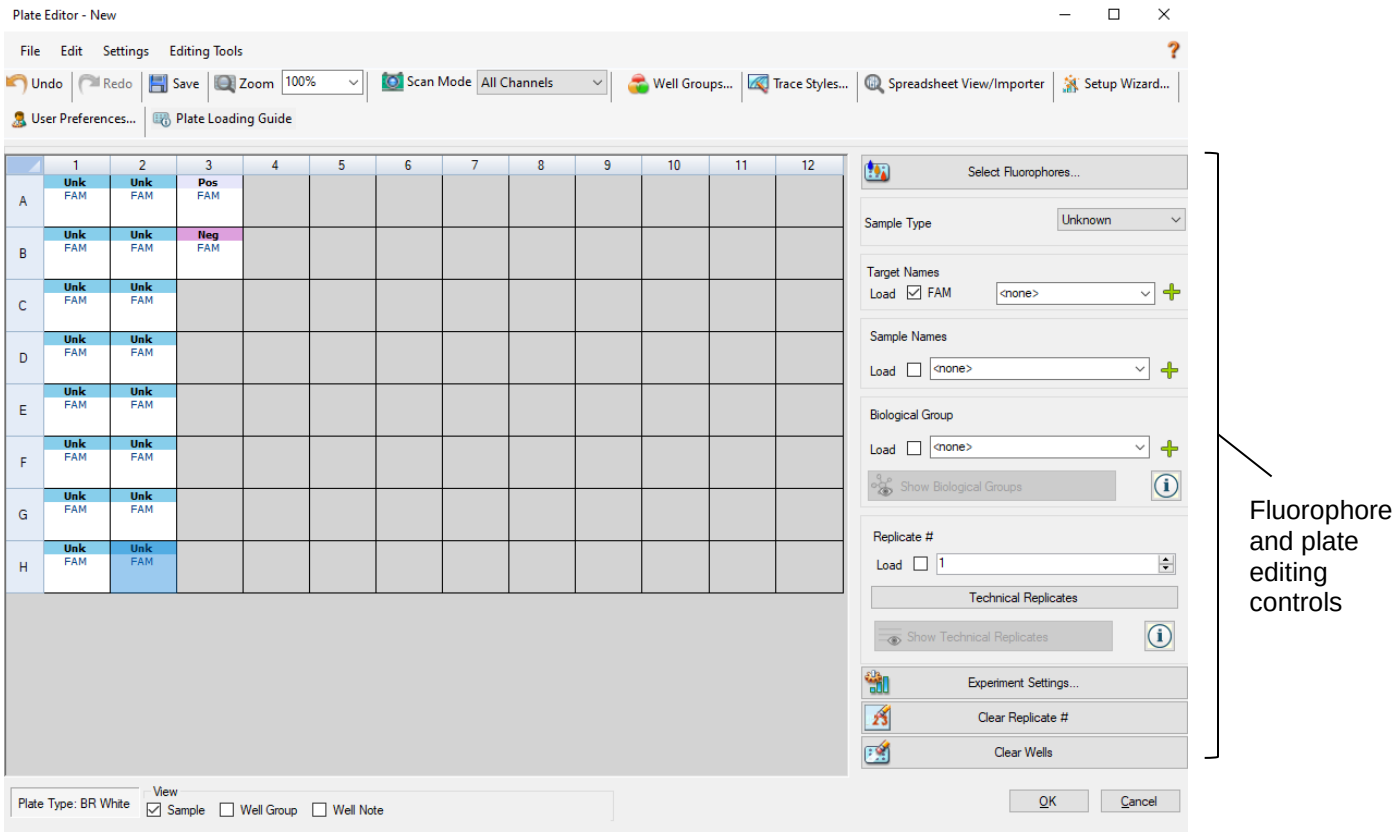


Fig. 5. Plate Editor. The fluorophore and plate editing controls are on the right.

10. Indicate which wells of the plate are used in your experiment, and whether they contain unknown samples, the positive controls, or the negative controls. Check the fluorophore box under Target Names.

11. Target names and sample names can also be added at this step.

12. Select **Start Run** in the Run Setup window to start the PCR run.

13. Run an existing protocol/plate: Click **Select Existing** from the Run Setup window to launch the file browser and load the previous saved files. Select **Start Run** to start the PCR run.

Refer to the CFX96 Touch Deep Well Instruction Manual for additional information.

C. Data Analysis

Data can be analyzed directly at the end of the PCR run or later by opening the stored data file. Follow instructions in the CFX96 Touch Deep Well Instruction Manual for opening data files and setting the data analysis parameters.

Interpreting results

Once the data analysis parameters have been set, results are interpreted by analyzing the Cq values of each sample.

Controls

- Verify the positive and negative controls before interpreting sample results.

    - **Negative Control:** The negative control monitors contamination. To perform a negative control, add distilled sterile water in place of the sample to be tested.

    - **Positive Control:** The positive control confirms that the experimental conditions are met for identifying a sample containing the target pathogen. A positive control is included with certain iQ-Design Assays and is recommended to use in your analysis. For iQ-Design Assays that do not include a positive control, a positive control will need to be created:

      To create a positive control, use target DNA (synthetic or isolated from a microorganism) in place of the sample. Adjust the DNA concentration of your positive control solution so that its amplification curve crosses the threshold after 18–32 PCR cycles ($18 \leq Cq \leq 32$).

      When working with a positive control prepared from a microbial culture, a minimum dilution of 2–3 logs is necessary.

      To prepare the dilution add 100 µl of DNA extract obtained from an overnight culture of a target strain in 9.9 ml of distilled sterile water (2 log). If the Cq of the sample obtained is too low ($Cq \leq 18$), dilute it further by taking 1 ml of this dilution and adding it to 9 ml of sterile distilled water (3 log). If the Cq of the sample is still too high ($Cq \geq 32$), use the undiluted sample.

      Note: Use caution when working with highly concentrated DNA samples, as they can easily contaminate your work area and can then be difficult to eliminate.

For the experiment to be valid, the controls must have the following results, as summarized in the table below. Otherwise, the PCR reaction must be repeated.

	Target*	Internal Control Detection**
Negative control	Cq = N/A	Cq ≥ 25
Positive control	18 ≤ Cq ≤ 35	Not significant

*For duplex or multiplex assays, there will be multiple target channels. Each target channel will need to meet the interpretation requirements listed in the table.
**For assays that do not include an internal control, refer to Section 6, Safety Precautions and Recommendations for Best Results, for performance verification with your specific matrix before analysis to ensure that amplification proceeds properly. A Cq value that is N/A in the target channel is a negative result.

Refer to Section 6, Safety Precautions and Recommendations for Best Results, for recommended procedures for positive and negative control result interpretation.

Samples

A **positive** iQ-Design Assay sample must have a Cq value ≥10 for the target.

- If the Cq is <10, verify that the raw data curve is a regular amplification curve (with a flat base line, followed by a rapid exponential increase of fluorescence and then a flattening out). If the curve seems correct, it may be considered a positive sample.
- If there is no Cq value (Cq = N/A) for the target and the internal control has a Cq ≥25, the sample is considered a **negative** sample.
- If the Cq value for the internal control is <25, it is not possible to interpret the result. Verify that the threshold was correctly placed, or that the curve as raw data is a regular amplification curve. If the curve does not have a characteristic shape, it will be necessary to repeat the PCR test.

For the experiment to be valid, the samples must have the following results, as summarized in the table below. Otherwise, the PCR reaction must be repeated.

Target*	Internal Control Detection**	Interpretation
Cq ≥ 10	Not significant	Positive
Cq = N/A	Cq ≥ 25	Negative
Cq = N/A	Cq = N/A	Inhibition

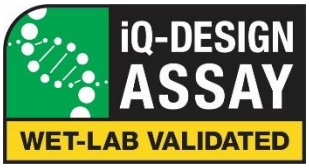
* For duplex or multiplex assays, there will be multiple target channels. Each target channel will need to meet the interpretation requirements listed in the table.
**For assays that do not include an internal control, refer to Section 6, Safety Precautions and Recommendations for Best Results, for performance verification with your specific matrix before analysis to ensure that amplification proceeds properly. A Cq value that is N/A in the target channel is a negative result.

Section 8

Test Performance and Validation

All iQ-Design Assays are developed and optimized following strict guidelines on specificity and sensitivity. Their performance is wet-lab validated for the following:

Amplification efficiency	90–110 %
Linear dynamic range	Minimum of six orders of magnitude
R^2	>0.98



Section 9

Revision History

Release date	Document number	Change
May 2021	10000142603 Ver A	New document
August 2021	10000142603 Ver B	Addition of legal disclaimer

Appendix — PCR Mix Calculation Guide

To find the correct volumes to use when preparing the PCR mix, add the total number of samples and controls to be analyzed and find the corresponding volumes of iQ-Design Assay Mix and iQ Multiplex Powermix in the table.

Total Number of Samples and Controls	iQ-Design Assay, µl	iQ Multiplex Powermix, µl	Total Number of Samples and Controls	iQ-Design Assay, µl	iQ Multiplex Powermix, µl	Total Number of Samples and Controls	iQ-Design Assay, µl	iQ Multiplex Powermix, µl
1	7.5	12.5	33	261	435	65	516	860
2	18	30	34	270	450	66	522	870
3	24	40	35	276	460	67	531	885
4	33	55	36	285	475	68	540	900
5	39	65	37	294	490	69	546	910
6	48	80	38	300	500	70	555	925
7	57	95	39	309	515	71	564	940
8	63	105	40	318	530	72	570	950
9	72	120	41	324	540	73	579	965
10	78	130	42	333	555	74	588	980
11	87	145	43	339	565	75	594	990
12	96	160	44	348	580	76	603	1005
13	102	170	45	357	595	77	612	1020
14	111	185	46	363	605	78	618	1030
15	117	195	47	372	620	79	627	1045
16	126	210	48	381	635	80	636	1060
17	135	225	49	387	645	81	642	1070
18	141	235	50	396	660	82	651	1085
19	150	250	51	405	675	83	657	1095
20	159	265	52	411	685	84	666	1110
21	165	275	53	420	700	85	675	1125
22	174	290	54	429	715	86	681	1135
23	180	300	55	435	725	87	690	1150
24	189	315	56	444	740	88	699	1165
25	198	330	57	453	755	89	705	1175
26	204	340	58	459	765	90	714	1190
27	213	355	59	468	780	91	723	1205
28	222	370	60	477	795	92	729	1215
29	228	380	61	483	805	93	738	1230
30	237	395	62	492	820	94	747	1245
31	246	410	63	498	830	95	753	1255
32	252	420	64	507	845	96	762	1270

Bio-Rad Laboratories, Inc.

Life Science Group

Website bio-rad.com **USA** 1 800 424 6723 **Australia** 61 2 9914 2800 **Austria** 00 800 00 24 67 23
Belgium 00 800 00 24 67 23 **Brazil** 4003 0399 **Canada** 1 905 364 3435 **China** 86 21 6169 8500
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