Cat. #NN0001

For Research Use

TaKaRa

Takara FFPE DNA QC All-in-One Kit

Product Manual

v202506Da

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I. Description

Formalin-fixed paraffin-embedded (FFPE) samples are widely used in both clinical and research settings, and analysis of FFPE samples has become more common in recent years. Although FFPE samples are useful for long-term storage, the formalin used in the fixation process causes chemical and physical modifications to nucleic acids and proteins, which can affect downstream assays (e.g., PCR amplification). Agarose gel electrophoresis is a method for evaluating DNA fragmentation, but it requires approximately 50 ng of DNA for measurement, making it unsuitable for evaluating limited or valuable samples. Although DNA concentration can be measured based on absorbance measurements, DNA fragmentation cannot be evaluated.

Takara FFPE DNA QC All-in-One Kit uses primers that amplify two amplicons, one long and one short, on a human housekeeping gene. This enables quantification of the number of effective molecules and evaluation of fragmentation by real-time PCR. PCR is performed using serially diluted standard DNA to create a standard curve, plotting the logarithm of the standard DNA copy number on the X-axis and the corresponding Ct values on the Y-axis. The standard curve is then used to quantify, based on the amplification of short amplicons, the number of effective molecules.

Since the amplification of long amplicons is reduced in highly fragmented FFPE-derived DNA, fragmentation of FFPE-derived DNA is evaluated by calculating the Long/Short Ratio from the quantitative values of long and short amplicons using the formula:

Long/Short Ratio = Long Assay quantitative value / Short Assay quantitative value

An unfragmented sample will have a Long/Short Ratio of 1.0. The more fragmented a sample is, the smaller the value will be.

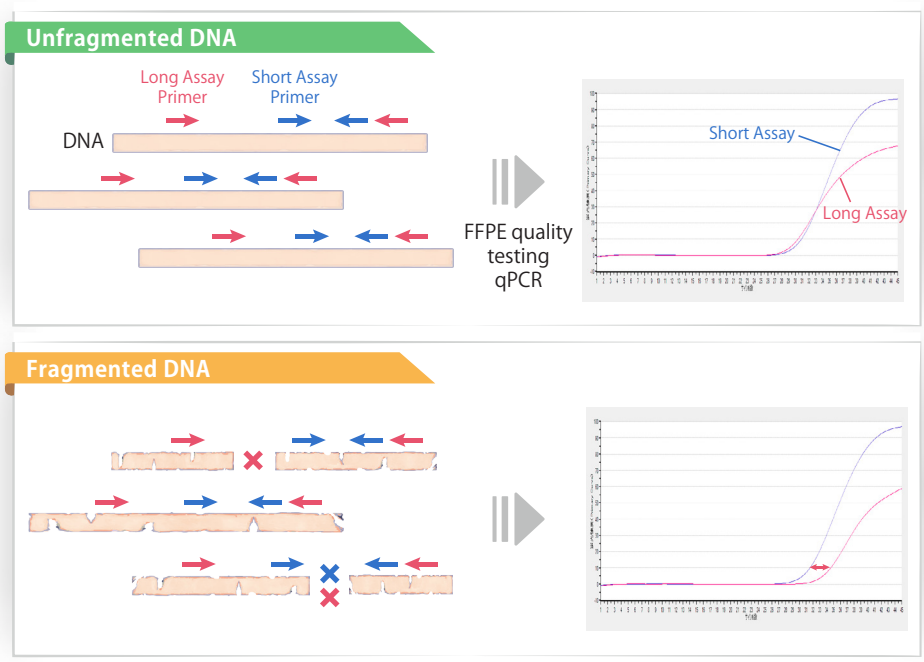

Fig. 1. Mechanism for evaluating DNA fragmentation

DNA fragmentation can be evaluated by comparing amplification with the Short Assay (92 bp), which amplifies a short amplicon, and the Long Assay (268 bp), which amplifies a long amplicon. Fragmented DNA reduces amplification of the Long Assay.

II. Components (100 reactions, 20 μl volume per reaction)

○	2X Premix FFPE QC*1	2X conc.	1 ml x2
●	Short Assay Primer/Probe Mix*2	10X conc.	200 μl
●	Long Assay Primer/Probe Mix*2	10X conc.	200 μl
ⓗ	RNase Free H_2O		1 ml x2
●	DNA Standard FFPE QC	3.3 x 10^5 copies/μl	100 μl
	EASY Dilution (for Real Time PCR)		1 ml x2
●	ROX Reference Dye II*3	50X conc.	100 μl

*1 Contains enzymes, matrix, etc.
*2 Contains fluorescent labeling probe; shield from light.
*3 Contains fluorescent substance; shield from light.

III. Storage -20℃

IV. Materials Required but Not Provided

[Instruments]

- Micropipette
- Micropipette tips (with hydrophobic filter)
- Real-time PCR tubes, etc.

[Equipment]

- Real-time PCR system (capable of detecting FAM)
  - Thermal Cycler Dice™ Real Time System IV (Cat. #TP1000/TP1010/TP1030)*
  - Thermal Cycler Dice Real Time System III (Cat. #TP950/TP970/TP980/TP990)*
  - CronoSTAR™ 96 Real-Time PCR System (Cat. #640231/640232)*
  - CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad)
  - LightCycler 96 System/ LightCycler 480 System II (Roche Diagnostics)
  - QuantStudio 5 Real-Time PCR System (96-well, 0.1 or 0.2 mL block) (Thermo Fisher Scientific)

  * Not available in all geographic locations. Check for availability in your area.

V. Precautions for Use

Read these precautions before use and follow them when using this product.

1. Since this is a product that measures human genomic DNA (gDNA), please be careful when handling it to avoid environmental contamination.
2. Accurate detection will not be possible if the primer or probe is degraded by nuclease contamination. Experimenters' sweat or saliva may contain nuclease, so be extremely careful when working.
3. Mix 2X Premix FFPE QC gently by inverting it to avoid creating bubbles, and homogenize the reagent before use. The reagent will not react properly unless it is mixed thoroughly.

   Note: Do not use a vortex to mix.
4. If 2X Premix FFPE QC is frozen and stored at -20°C, precipitation may occur during storage. Warm it gently with your hands or leave it at room temperature for a few minutes to thaw, then mix by inverting it until it dissolves completely.
5. Keep reagents on ice when preparing the reaction solution. When preparing or dispensing the reaction solution, be sure to use a new disposable tip each time to prevent sample contamination.
6. This product performs amplification and detection in real time, so it is not necessary to analyze the amplification products with electrophoresis, etc., after the reaction ends. Do not remove the amplification products from the tube, as this could cause contamination with nucleic acid in the laboratory.

VI. Protocol

1. Preparation of FFPE-derived genomic DNA samples

Dilute each gDNA sample to 1 ng/μl with EASY Dilution (for Real Time PCR).

2. Preparation of standard samples for standard curve creation

Use DNA Standard FFPE QC to prepare five serial diluents ranging from 3.3 x 10^3 copies/μl to 13 copies/μl.

Caution: Be sure to prepare DNA diluents just before use.

1) Dispense 45 μl of EASY Dilution (for Real Time PCR) into six 1.5 ml microtubes.
2) Add 5 μl of ● DNA Standard FFPE QC (3.3 x 10^5 copies/μl) to one of the tubes from Step 1), mix well, and centrifuge briefly to prepare a diluted solution of 3.3 x 10^4 copies/μl.
3) Add 5 μl of the 3.3 x 10^4 copies/μl solution prepared in Step 2) to another 45 μl of fresh EASY Dilution (for Real Time PCR) prepared in Step 1. Mix well, and centrifuge briefly to prepare 3.3 x 10^3 copies/μl.
4) Add 15 μl of the 3.3 x 10^3 copies/μl solution prepared in Step 3) to another 45 μl of EASY Dilution (for Real Time PCR) prepared in Step 1. Mix well, and centrifuge briefly to prepare 8.25 x 10^2 copies/μl. Repeat the dilution procedure to prepare serial dilutions down to 13 copies/μl.

No.	Diluent concentration	Diluent preparation method
1	3.3 x 10^4 copies/μl	5 μl of DNA Standard FFPE QC stock solution + 45 μl of EASY Dilution
2	3.3 x 10^3 copies/μl	5 μl of 3.3 x 10^4 copies/μl solution from No. 1 + 45 μl of EASY Dilution
3	8.25 x 10^2 copies/μl	15 μl of 3.3 x 10^3 copies/μl solution from No. 2 + 45 μl of EASY Dilution
4	2.06 x 10^2 copies/μl	15 μl of 8.25 x 10^2 copies/μl solution from No. 3 + 45 μl of EASY Dilution
5	52 copies/μl	15 μl of 2.06 x 10^2 copies/μl solution from No. 4 + 45 μl of EASY Dilution
6	13 copies/μl	15 μl of 52 copies/μl solution from No. 5 + 45 μl of EASY Dilution

Note: Reactions will be performed using No. 2–6 (3.3 x 10^3 copies/μl to 13 copies/μl) of the six serial diluents above as standard samples for creating a standard curve. Use 2 μl of each per reaction. n = 2 reactions are recommended.

3. Real-time PCR reaction

1) Preparation of reaction solution

a. Prepare two master mixes, one for the Short Assay and one for the Long Assay, using the composition in the tables below.

For each, prepare the required number of components excluding the sample, calculated as (number of standards for the standard curve + number of samples + NTC) x 1.1.

b. Dispense 18 μl into each reaction tube or plate and gently cap or seal.

Caution: Do not touch the tubes, plates, lids, or seals with bare hands as this may cause fluorescence noise.

【When ROX Reference Dye is not used*1 】

< Short Assay >

[Per reaction]

Reagent	Volume
○ 2X Premix FFPE QC	10.0 μl
● Short Assay Primer/Probe Mix	2.0 μl
(H2O) RNase Free H_2O	6.0 μl
Sample (or standard sample for creating a standard curve or (H2O) RNase Free H_2O)	2.0 μl
Total	20.0 μl

< Long Assay >

[Per reaction]

Reagent	Volume
○ 2X Premix FFPE QC	10.0 μl
● Long Assay Primer/Probe Mix	2.0 μl
(H2O) RNase Free H_2O	6.0 μl
Sample (or standard sample for creating a standard curve or (H2O) RNase Free H_2O)	2.0 μl
Total	20.0 μl

*1 Applicable models

- Thermal Cycler Dice Real Time System series (Cat. #TP1000/TP950, etc.)
- CronoSTAR 96 Real-Time PCR System (Cat. #640231/640232)
- CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad)
- LightCycler 96 System/LightCycler 480 System II (Roche Diagnostics)

【When using ROX Reference Dye*2】

< Short Assay >

[Per reaction]

Reagent	Volume
○ 2X Premix FFPE QC	10.0 μl
● Short Assay Primer/Probe Mix	2.0 μl
● ROX Reference Dye II	0.4 μl
Ⓗ RNase Free H_2O	5.6 μl
Sample (or standard sample for creating a standard curve or Ⓗ RNase Free H_2O)	2.0 μl
Total	20.0 μl

< Long Assay >

[Per reaction]

Reagent	Volume
○ 2X Premix FFPE QC	10.0 μl
● Long Assay Primer/Probe Mix	2.0 μl
● ROX Reference Dye II	0.4 μl
Ⓗ RNase Free H_2O	5.6 μl
Sample (or standard sample for creating a standard curve or Ⓗ RNase Free H_2O)	2.0 μl
Total	20.0 μl

*2 Applicable models

- QuantStudio 5 Real-Time PCR System (96-well, 0.1 or 0.2 mL block) (Thermo Fisher Scientific)

2) Addition of sample (template)

Add 2 μl of Ⓗ RNase Free H_2O (Negative Control), standard samples for creating a standard curve (3.3×10^3 copies/μl, 8.25×10^2 copies/μl, 2.06×10^2 copies/μl, 52 copies/μl, 13 copies/μl), and sample DNA to the tubes or plates and cap or seal tightly.

3) Performing real-time PCR

Perform real-time PCR reaction under the following conditions.

[Cautions]

- Before the reaction, spin down the tubes or plates to make sure that no reaction solution adheres to the inner wall and there are no air bubbles. Air bubbles may affect fluorescence detection.
- Turn the real-time PCR apparatus on in advance to preheat the lid.

< Reaction conditions >

Hold

95℃ 30 sec

PCR：45 cycles

95℃ 5 sec

60℃ 60 sec (fluorescence detection: FAM)

Note: For the Thermal Cycler Dice Real Time System IV/III, please select "Fast" for the Speed setting on the Thermal Profile Setup screen. When analyzing data, ensure that the normalization correction setting is turned OFF. For instructions on how to change the normalization correction setting, please refer to the user manual for the instrument.

VII. Quality Assessment

1. After the reaction is complete, confirm that the amplification curve and analysis parameters are appropriate* and calculate the Ct value.

   * Refer to the manufacturer's manual for your real-time PCR instrument.

   < Fluorescence detection filter >

Assay type	Fluorescence detection filter
Short Assay	FAM
Long Assay	FAM

2. To create the standard curves, plot the logarithm value for the number of copies of the standard sample (DNA Standard FFPE QC serial dilution solution) on the X-axis and the Ct value at that concentration on the Y-axis for the Short Assay and the Long Assay, respectively.

   < Points to consider when evaluating standard curves >

   There are two points to consider when evaluating standard curves: slope and linearity. PCR amplification efficiency can be calculated from the slope.
   The optimum range is considered to be 80 to 120%. The amplification efficiency can be calculated using the following formula.

   $$\text{Amplification efficiency (E)} = 10^{[-1/\text{slope}]} - 1$$

   (when initial template concentration (Log_{10}) is on the X-axis, and Ct value is on the Y-axis)

   Linearity is evaluated using the coefficient of determination (R^2), and ideally the value should be ≥0.98.

3. Use the standard curve from Step 2 to calculate the quantitative values (copies/reaction) of the Short Assay and Long Assay from the Ct value of each sample to be measured and the quantitative average value (n = 2).

4. Calculate the effective number of molecules (copies/μl) in each FFPE DNA sample from the quantitative average value (from Step 3) of the Short Assay.
   - Divide the quantitative average value by 2 μl (the amount added to each qPCR reaction).
   - Multiply by the dilution factor used in Step 1 of Section VI to calculate the number of effective molecules (copies/μl).

5. Calculate the fragmentation rating (Long/Short Ratio) from the quantitative average value of both the Short and Long Assays (from Step 3).

   Long/Short Ratio = (Long Assay quantitative average value) / (Short Assay quantitative average value)

   Note: The Long/Short Ratio is evaluated on a scale of 0 to 1, with a value closer to 1 indicating high-quality DNA (unfragmented DNA).

VIII. Related products

Thermal Cycler Dice™ Real Time System IV (Cat. #TP1000/TP1010/TP1030)*
Thermal Cycler Dice™ Real Time System III (Cat. #TP950/TP970/TP980/TP990)*
CronoSTAR™ 96 Real-Time PCR System (Cat. #640231/640232)*
NucleoSpin DNA FFPE XS (Cat. #740980.10/.50/.250)*

* Not available in all geographic locations. Check for availability in your area.