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KRISHGEN® Residual Plasmid DNA Quantitation Real Time PCR Kit

Instruction Manual

REF **KBPL9091**

Store the kit and its components at -20ºC

For Research Use Only **RUO**

100 reactions

Introduction:

Residual Plasmid DNA Quantitation Real Time PCR Kit is used to quantitate plasmid DNA residues in gene therapy products, such as the plasmid DNA of lentiviral vector preparation in CAR-T cell therapy. This kit uses duplex real-time PCR technology to detect trace amounts of residual plasmid DNA by means of consensual plasmid sequences, such as replicon of ColE1/pMB1/pBR322/pUC. The target gene (FAM) performs rapid, specific, and reliable quantitative determination of the 102 copies/ul level of residual Plasmid DNA. IPC−Internal Positive Control (VIC) is included in the Plasmid Primer & Probe MIX to evaluate the performance of each PCR reaction. Customers can send DNA sequence of empty plasmid backbones to our technicians for confirmation in advance.

Intended Use:

The KRISHGEN® Residual Plasmid DNA Quantitation Real Time PCR Kit can be used to quantitate Residual Plasmid residual DNA in different stages of biopharmaceutical products, from in-process samples to final products.

Kit Components:

1. Plasmid DNA Control (lyophilized) – 1 vial
2. Plasmid Primer & Probe Mix – 500 ul x 1 vial
3. qPCR Master Mix – 850 ul x 2 vials
4. DNA Dilution Buffer – 1.5 ml x 3 vials
5. Instruction Manual

Additional Requirements to be provided by the End-user:

1. DNase-free, Low Retention Microfuge Tubes, 1.5ml
2. Low Retention Tips of 1000 ul, 100 ul, 10 ul
3. 96-well qPCR plates with sealing film or PCR 8-strip tubes with caps
4. Microcentrifuge
5. Vortex mixer
6. Micropipettes of 1000 ul, 100 ul, 10 ul
7. Real-time PCR instrument
8. Microplate shaker

Applied instruments, including but not limited to the following:

1. ABI 7500 Real-Time PCR System
2. CFX96 Real-Time PCR System
3. StepOne Plus Real-Time PCR System
4. Real Time PCR System, compatible

Stability and Storage:

Store the DNA Control, qPCR Mix and DNA dilution buffer at -20ºC, protect from light.

General Precautions:

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use a new tip for every procedure.
- Store all extracted positive material (specimens, controls and amplicons) away from all other reagents and add it to the reaction mix in a distantly separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.

- Use disposable protective gloves and laboratory cloths, and protect eyes while samples and reagents handling. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local regulations.
- Samples should be considered potentially infectious and handled in biological cabinet in compliance with appropriate biosafety practices.
- Clean and disinfect all samples or reagents spills using a disinfectant, such as 0.5 % sodium hypochlorite or another suitable disinfectant.
  Avoid samples and reagents contact with the skin, eyes, and mucous membranes. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice immediately.
- Safety Data Sheets (SDS) is available on request.
- Use of this product should be limited to personnel trained in DNA amplification techniques.
- Workflow in the laboratory must be one-directional, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where the previous step was performed.
  Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

Reagent Preparation:

DNA Standard serial dilutions for the standard curve:

- Spin Plasmid linear DNA Control for 15 seconds in a centrifuge and then add 55 ul of ddH_2O accurately to the bottom of the tube to dissolve the lyophilized powder.
- Gently flick the Plasmid linear DNA Control standard solution with finger several times, then spin for 3-5 seconds in a centrifuge. Repeat 3 times to fully dissolve the lyophilized powder in the solution.
- Please check the concentration on the label of the tube containing the Plasmid linear DNA Control prior to dilution.

a. Thaw Residual Plasmid DNA Control and DNA Dilution Buffer completely at 2-8ºC or melt on ice. Vortex to mix well and quickly spin down the reagents for 3-5 seconds in microcentrifuge. Repeat this 3 times.
b. Label eight nonstick 1.5 ml centrifuge tubes: A, B, C, ST1, ST2, ST3, ST4 and ST5.
c. Dilute the Residual Plasmid DNA Control to 4.97×10^8 copies/ul with DNA Dilution Buffer (DDB) in the A tube. Vortex to mix well and quickly spin down the reagents for 3-5 seconds in microcentrifuge and repeat 3 times.
d. Add 90 ul DNA Dilution Buffer to all tubes B, C, ST1, ST2, ST3, ST4 and ST5.
e. Perform the serial dilutions according to below table.

Standard Concentration Dilution	Standard Vial	Dilution Particulars
4.97×10^8	A	Dilute the DNA control with DNA Dilution Buffer
4.97×10^7	B	10 ul A + 90 ul DNA Dilution Buffer
4.97×10^6	C	10 ul B + 90 ul DNA Dilution Buffer
4.97×10^5	ST1	10 ul C + 90 ul DNA Dilution Buffer
4.97×10^4	ST2	10 ul ST1 + 90 ul DNA Dilution Buffer
4.97×10^3	ST3	10 ul ST2 + 90 ul DNA Dilution Buffer
4.97×10^2	ST4	10 ul ST3 + 90 ul DNA Dilution Buffer
4.97×10^1	ST5	10 ul ST4 + 90 ul DNA Dilution Buffer

Note:

- *The remaining unused DNA Dilution Buffer need to be stored at 2-8℃. If the solution is cloudy or contains precipitates, heat at 37℃ until it is clear.*
- *At least five concentration of standard curve should be included. To select appropriate sample dilutions, we recommend performing method validation before sample testing.*

Sample Preparation:
Test Sample Preparation:
Take 100 ul of the test sample and add to a new 1.5 ml microfuge tube.

Extraction Reference Control (ERC) samples Preparation
According to the Residual Plasmid DNA spike concentration in ERC samples (Take the samples containing $4.97x10^5$ copies of Residual Plasmid DNA as example), specific preparation procedure is as follows:
(1) Take 100 ul of the test sample and add it to a new 1.5 ml microfuge tube.
(2) Add another 10 ul of ST2 solution and mix thoroughly, lable as ERC sample.

Negative Control Sample (NCS) Preparation:
Add 100 ul of DDB to a new 1.5 ml microcentrifuge tube, and label as NCS.

Assay Protocol:

A) qPCR Mix preparation:

1. Determine the number of reaction wells based on the standard curve, with the number of test samples and control samples. Generally, triplicates are tested for each sample.

Number of reaction wells = (5 standard points on the standard curve + 1 NTC + 1 NCS + test samples) x 3

2. Prepare qPCR MIX according to the number of reaction wells.

Reagent	Volume / reaction	Volume for 30 reaction (includes 10% coverage)
qPCR Master Mix	15 ul	495 ul
Plasmid Primer&Probe Mix (Incl IPC)	5 ul	165 ul
Total Volume	20 ul	660 ul

3. After thoroughly mixing qPCR MIX, follow 20 ul each tube is divided into PCR 8-strip tubes or 96-well qPCR plate.

B) qPCR Reaction mix preparation:

1. Prepare qPCR Reaction mix according to Table below, and a 96-well plate layout template is shown in chart.

Tubes	Standard Curve	NTC	NCS	Test sample	Test Sample ERC
qPCR Mix	20 ul	20 ul	20 ul	20 ul	20 ul
Samples	10 ul ST1 – ST5	10 ul DDB	10 ul purified NCS	10 ul purified test sample	10 ul purified ERC sample
Total Volume	30 ul	30 ul	30 ul	30 ul	30 ul

Chart: Reference 96-well plate layout.

NTC		S1	S1	S1	S1 ERC	S1 ERC	S1 ERC		ST5	ST5	ST5	A
NTC		S2	S2	S2	S2 ERC	S2 ERC	S2 ERC		ST4	ST4	ST4	B
NTC		S3	S3	S3	S3 ERC	S3 ERC	S3 ERC		ST3	ST3	ST3	C
		S4	S4	S4	S4 ERC	S4 ERC	S4 ERC		ST2	ST2	ST2	D
NCS		S5	S5	S5	S5 ERC	S5 ERC	S5 ERC		ST1	ST1	ST1	E
NCS												F
NCS												G
												H
1	2	3	4	5	6	7	8	9	10	11	12	

This example represents the assay for a standard curve with 5 concentration gradients (ST1-ST5), 1 NTC, 1 NCS, 5 test samples (S1 to S5), and 5 ERC samples (S1 ERC to S5 ERC), with 3 replicates for each sample.

In specific testing, the plate layout for sample loading can be adjusted based on the sample quantity. Please refer to the example shown in chart.

Seal the 96-well plate with sealing film. Mix well in microplate shaker, then spin down the reagents for 10 seconds in microcentrifuge and place it on the qPCR instrument.

C) qPCR Program Settings:

NOTE: The following instructions apply only to the ABI7500 instrument with SDS v1.4. If you use a different instrument or software, refer to the applicable instrument or software documentation.

1. Create a new document, and then in the Assay drop-down list, select Standard Curve (Absolute Quantitation).
2. In the Run Mode drop-down list, select **Standard 7500**, and then click **next**.
3. Click New Detector:
   a. Enter Residual Plasmid DNA in the name field.
   b. Select **FAM** in the reporter Dye drop-down list and select (**none**) in the Quencher Dye drop-down list, and then click **OK.**
   c. Select a color for the detector, then click **Create another.**
4. Click **New Detector**:
   a. Enter IPC in the name field.
   b. Select **VIC** in the reporter Dye drop-down list and select (**none**) in the Quencher Dye drop-down list, and then click **OK.**
   c. Select a color for the detector, then click **OK.**
   d. Select the detectors, then click **Add** to add the detectors to the document.
5. Select **ROX** as the passive reference dye, and then click **next.**
6. Select the applicable set of wells for the samples, and then select the Residual Plasmid DNA detector and IPC detector for each well.
7. Select Finish, and then set thermal-cycling conditions:
   a. Set the thermal cycling reaction volume to 30 ul.
   b. Set the temperature and time as following:

qPCR running temperature and time:

Step	Temp.	Time (mm:sec)	Cycles
Activation	95ºC	10:00	1
Denaturation	95ºC	00:15	40
Annealing/extension	60ºC*	01:00	

*Instrument will read the fluorescence signal during this step.

8. Save the document, then click **Start** to start the real time qPCR run.

NOTE: The following instructions apply only to the ABI7500 instrument. If you use a different instrument or software, please contact us at sales1@krishgen.com.

Calculation of Results:

1. Select **Set up** tab, then set tasks for each sample type by clicking on the Task Column drop-down list:
   a. NTC: target DNA detector task = **NTC**
   b. NCS, test samples, and ERC wells: target DNA detector task = **Unknown**

2. Set up the standard curve as shown in table:

Settings for Standard curve

Tube label	Task	Quantity (copies/ul)
ST1	Standard	$4.97x10^5$
ST2	Standard	$4.97x10^4$
ST3	Standard	$4.97x10^3$
ST4	Standard	$4.97x10^2$
ST5	Standard	$4.97x10^1$

3. Select the **Results** tab, and then select Amplification Plot.

4. In the Data drop-down list, select **Delta Rn vs Cycle**.

5. In the Analysis Settings window, enter the following settings:
   a. Select **Manual Ct**.
   b. In the Threshold field, Residual Plasmid DNA enter 0.05 and IPC enter 0.1.
   c. Select **Automatic Baseline**.

6. Click the button  in the toolbar, and then wait the plate analyzing.

7. Select the **Result** tab> >**Standard curve** tab, then verify the Slope, Intercept and R^2 values.

8. Select the Report tab, and then achieve the mean quantity and standard deviation for each sample.

9. Select **File > > Export > > Results**. In the Save as type drop-down list, select **Results Export Files**, then click **Save**.

10. In the Report panel of Results, the 'Mean Quantity' column can read the detection values of NTC, NCS, test sample, and ERC sample, in copies/ul.

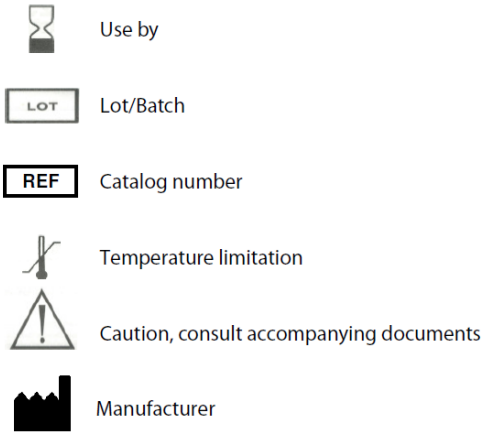
11. The recovery rate of ERC samples is calculated based on the value of test samples and the ERC samples. The recovery rates should be between 50% and 150%.

12. The Ct value of NCS should be larger than the mean Ct value of the lowest concentration in the standard curve, and it shows normal amplification curve in the VIC signal channel.

13. The Ct value of NTC should be 2 larger than the Ct value of ST5, or set specific standards based on the laboratory's own validation results, and it shows normal amplification curve in the VIC signal channel.

Note: The parameter settings of the result analysis should be configured on the specific model and the software version, and generally can also be automatically interpreted by the instrument.

Symbols



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