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1.0 Purpose

This procedure describes the materials and methods for quantitative PCR (qPCR) amplification and detection of residual plasmid DNA (Kanamycin resistance gene) that may be present in a sample. The analysis is performed using the resDNASEQ Quantitative plasmid DNA Kanamycin resistance gene kit. An internal positive control (IPC) is included in the kit reagents to evaluate the performance of each reaction.

2.0 Scope

The resDNASEQ Quantitative plasmid DNA kit is designed to detect residual kanamycin resistance plasmid in a sample. The kit is able to detect residual kanamycin resistance gene DNA to a lower limit of 15 copy per reaction (1.5 copy/µL)

3.0 Authority and Responsibility

3.1 Director, Process Analytics/Quality Control (PA/QC),

- Defines this procedure.

3.2 PA/QC Personnel

- Performs of this procedure.
- Reviews the data and documentation of the results.

4.0 Equipment, Materials and Reagents

4.1 resDNASEQ Quantitative plasmid DNA Kanamycin resistance gene Kit (BDP PN 31401)

4.2 DNA dilution buffer (included in the kit, or BDP PN 31407 if purchased separately)

4.3 MicroAmp Optical 96 Well Reaction Plates (BDP PN 21141 or BDP-approved equivalent)

4.4 MicroAmp Optical Adhesive Film (BDP PN 21142 or BDP-approved equivalent)

4.5 1X TE (BDP PN 30267 or BDP-approved equivalent)

4.6 Microcentrifuge tubes, Low binding (1.7 mL) (BDP PN 31129 or BDP-approved equivalent)

4.7 1X PBS (BDP PN 30007 or BDP-approved equivalent)

4.8 Roche MagNA Pure 24 Total NA Isolation Kit (BDP PN 31236) or QIAGEN DNeasy Blood and Tissue Kit (BDP PN 30443)

4.9 QuantStudio 6 Real-Time PCR system or QuantStudio 3 Real-Time PCR System

4.10 Roche MagNA Pure 24, Nucleic Acid extraction Instrument

5.0 Procedure

5.1 Determine the sample composition to determine whether a nucleic acid extraction is required.

5.1.1 Previously purified DNA in low salt (<100 mM) typically does not require extraction prior to amplification.

5.1.2 Samples containing concentrated protein, intact cells and/or high salt or other PCR inhibitors must be extracted prior to amplification.

5.2 An extraction negative control will be performed in which 200 µL of 1X PBS are extracted in parallel with the test samples.

5.3 Extract all test samples and the extraction negative control according to ***SOP 22972 – Operation and Maintenance of the MagNA Pure 24 System for Nucleic Acid Extractions or SOP 22212 – Purification of DNA Using the DNeasy Blood and Tissue Kit***.

5.4 qPCR Master Mix Preparation

5.4.1 Completely thaw the resDNASEQ Quantitative plasmid DNA Kanamycin Resistance Gene kit reagents.

5.4.2 Record the reagent and material part numbers, lot numbers and expiration dates as well as any relevant additional comments on **Form 23002-01**.

5.4.3 Briefly vortex the reagents.

5.4.4 Prepare a master mix as follows:

Kit Reagent	Volume for 1 Reaction (µL)
Negative Control (Water)	2.0
10X DNA Assay Mix	3.0
2X Environmental Master Mix	15.0
Total Master Mix Volume	20.0

NOTE: For qPCR reaction, 10 µl of test sample will be added on the plate, which makes total 30 µl volume per reaction.

5.5 Prepare a sufficient volume of master mix to account for all test samples and the extraction negative control with 10% excess to account for losses due to repeated pipetting.

5.6 Record the volumes used on **Form 23002-02**.

5.7 Mix the master mix by pipetting up and down.

5.8 Add 20 µL of master mix to each well of the 96-well PCR reaction plate that will be used.

5.9 Include three No Template Control (NTC) wells on the plate in which 20 µL of master mix are combined with 10 µL of Negative Control (Water) from the resDNASEQ kit.

5.10 Temporarily seal the plate with adhesive film and store in the refrigerator until template addition.

5.11 Preparation of the Standard Curve

5.11.1 Thaw the resDNASEQ Kanamycin resistance gene DNA control tube and DNA Dilution Buffer (DDB) completely.

5.11.2 Prepare a set of standard curve samples as follows:

Serial Dilution Tube	Dilution Instructions	Concentration in Final PCR Reaction
Control (provided in kit, 3.0 x 10^7 copy number/ µL)	DNA Control Tube	N/A
Dilution 1 (3.0 x 10^5 copy number/ µL)	10 µL DNA control + 990 µL DDB	N/A
SD1	20 µL Dilution 1 + 180 µL DDB	300,000 copy number / reaction
SD2	20 µL SD1 + 180 µL DDB	30,000 copy number / reaction
SD3	20 µL SD2 + 180 µL DDB	3,000 copy number / reaction
SD4	20 µL SD3 + 180 µL DDB	300 copy number / reaction
SD5	20 µL SD4 + 180 µL DDB	30 copy number / reaction
SD6	100 µL SD5 + 100 µL DDB	15 copy number / reaction

5.12 Sample addition to the qPCR plate

5.12.1 Extraction Negative Control: Add 10 µL of Extraction Negative Control to triplicate wells of the plate.

5.12.2 Test sample(s): Add 10 µL of the unknown sample to triplicate (GLP/GMP test) or duplicate (R&D test) wells of the plate.

NOTE: It may be necessary to dilute the sample so that the result falls within the standard curve.

5.12.3 **Standard Curve**: Add 10 µL of each standard curve dilution to the plate in triplicate (GLP/GMP) or duplicate (R&D test) wells of the plate.

5.12.4 Seal the plate with adhesive film. The plate may be kept at room temperature or in the refrigerator for up to 30 minutes prior to amplification on a qPCR instrument.

5.13 Place the sealed plate into the qPCR instrument ensuring that the plate is appropriately placed in the carriage by lining up the notched corner of the plate to the notched corner of the plate holder.

5.14 Set up the thermal cycler to detect FAM (KanR DNA) and VIC (IPC control).

5.15 Program the thermal cycler to run the following program:

Temperature	Time (min: sec)	Cycles
95°C	10:00	1
95°C	00:15	40
60°C	01:00	

5.16 Upon completion of the qPCR run, unload, and discard the plate. Do not remove the adhesive cover to prevent laboratory contamination with the target amplicon!

5.17 Analyze the KanR-FAM results using a manual Ct threshold of 0.1 or automatic and use an automatic threshold for the IPC-VIC results.

NOTE: Use of other Ct thresholds may be reported with supervisor approval.

6.0 Validity of the Assay

6.1 Record the assay control results on **Form 23002-03**.

6.2 For the assay to be considered valid, the following criteria must be met:

6.2.1 KanR -FAM

6.2.1.1 The NTC wells must either fail to generate an amplification product (Undetermined or No Ct) or the mean Ct of the NTC wells must be ≥ the mean Ct for the lowest standard curve point on the plate (SD6, 15 copy/reaction).

6.2.1.2 The Extraction Negative Control wells must either fail to generate an amplification product (Undetermined or No Ct) or the mean Ct for the Extraction Negative Control wells must be ≥ the mean Ct for the lowest standard curve point on the plate (SD6, 15 copy/reaction).

6.2.1.3 The slope of the standard curve must be ≥-3.7 and ≤-3.1.

6.2.1.4 The R2 value for the standard curve must be ≥0.950

6.2.2 Internal Positive Control (IPC)-VIC

6.2.2.1 The mean IPC-VIC Ct for the sample must be within 3.33 cycles of the mean Ct for the NTC replicates.

NOTE: A sample with a mean Ct for IPC-VIC that is >3.33 cycles higher than the mean Ct for the NTC replicates would indicate PCR inhibition and should be re-extracted.

6.3 If the run is valid, proceed to step 7.1 to determine the amount of residual KanR plasmid present in the unknown sample(s).

6.4 If the run is invalid, the assay may be repeated with supervisor approval and indicate in the Comments section of **Form 23002-03** that the assay was repeated.

7.0 Analysis of Results

7.1 Record the assay data on **Form 23002-04**.

7.2 Any dilution or concentration factors must be accounted for to determine the concentration of the original stock. For example, if 200 µL of sample were extracted on the MagNA Pure 24 and were eluted in 100 µL, then the concentration determined for the sample should be divided by a factor of 2.

7.3 Divide the concentration in pg/reaction by 10 to determine the concentration in copy/µL (since 10 µL of template were analyzed in the qPCR).

7.4 If the concentration of residual host cell DNA exceeds the standard curve, it may be necessary to repeat the assay with a diluted sample (dilute in 1X TE).

8.0 Documentation

8.1 Record all reagent and material part numbers, lot numbers and expiration dates on **Form 23002-01**. Record the 96-well plate layout and master mix preparation volumes on **Form 23002-02**. Record control results on **Form 23002-03** Record the qPCR results on **Form 23002-04**.

9.0 References and Related Documents

SOP 22972 *Operation and Maintenance of the MagNA Pure 24 System for Nucleic Acid Extractions*

SOP 22212 *Purification of DNA Using the DNeasy Blood and Tissue Kit*

Form 23002-01 *quantitative PCR reagents*

Form 23002-02 *96 well qPCR plate key*

Form 23002-03 *qPCR assay validity analysis*

Form 23002-04 *qPCR data*