Porcine Detection Kit

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Protocol

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In this Guide...

This document describes how to use the Porcine Detection Kit to extract genomic DNA from a food sample or other product and test it for the presence of porcine DNA using QPCR analysis.

1 Before You Begin

Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

2 Procedures

This chapter contains instructions on how to extract and isolate DNA from test samples and perform QPCR amplification with the isolated DNA. It also provides guidelines on analysis of the QPCR results to determine if porcine DNA is present in the test samples.

3 Troubleshooting Guide

This chapter offers recommendations for dealing with potential issues that may arise when using the Agilent Porcine Detection kit.

4 Appendix

This chapter contains instructions on decontaminating the immersion blender used for sample homogenization to prevent cross-contamination between samples.

Contents

1
Before You Begin

Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

Kit Contents

Materials Provided with the Agilent Porcine Detection Kit

DNA Isolation Kit, Part Number 5500-0051 (Boxes 5972-3629 and 5972-3630), 50 preparations	
Nucleic Acid Binding Buffer	25 ml
High Salt Wash Buffer	24 ml
Elution Buffer (10 mM Tris-HCl, pH 7.5)	12 ml
DNA-Binding Spin Cups and 2-ml Receptacle Tubes	50 each
1.5-ml Collection Tubes	50 each
Proteinase K	2 × 0.5 ml
Proteinase K Digestion Buffer	2 × 5 ml
Agilent Porcine Detection QPCR Kit, Part Number 5190-3741, 400 reactions	
Porcine Detection QPCR Master Mix (2×)	2 × 2.5 ml
Porcine DNA Control (1000×)	50 µl
Porcine Detection Assay Mix (10×)	1 ml

Storage Conditions

Store the proteinase K and proteinase K digestion buffer at 4°C. Store the remaining DNA isolation kit components at room temperature.

Store the QPCR reagents kit at –20°C upon receipt. After thawing, store the QPCR master mix at 4°C. Once thawed, full activity of the master mix is guaranteed for 6 months. For the remaining QPCR reagents, continue storage at –20°C.

Avoid thawing and refreezing the porcine detection assay mix >10 times. If necessary, aliquot the assay mix into smaller volumes and then freeze at –20°C. Thaw single aliquots as needed.

The porcine detection assay mix is light sensitive; keep the stock and any solutions containing the assay mix protected from light whenever possible.

Required Equipment, Supplies and Reagents

Additional Equipment, Supplies and Reagents Required
Equipment for food and beverage homogenization:
Handheld immersion blender or disposable pellet pestles with attachable spinning motor (see "To prepare the test samples" on page 16 for discussion on these two homogenization techniques)
100% ethanol, 200 proof (USP grade or equivalent)
Sterile, nuclease-free water
Micropipettors with compatible aerosol-resistant tips, sizes: 20 µl, 20 µl and 1000 µl (2 sets of micropipettors are preferable - one for DNA extraction/isolation and one for QPCR set up)
Disposable pipettes for preparing 80% ethanol solution
Incubator or water bath set to 65°C
Sterile glass bottle or polypropylene tube (e.g. 14-ml BD Falcon polypropylene round-bottom tubes or 50-ml BD Falcon polypropylene conical tubes)
Vortex mixer
Spectrofluorometric thermal cycler, e.g. the Agilent Mx3000P or Mx3005P QPCR System
QPCR tubes, strip tubes or plates with covers
Microcentrifuge with rotor for 1.5-ml microcentrifuge tubes, e.g. the Eppendorf 5414D with aerosol-tight rotor
1.5-ml microcentrifuge tubes, sterile (preferably low-adhesion)
Paper towels
Disposable gloves (latex or nitrile)
Electronic balance (accurate in the 25 to 250 mg range)
Disposable lab coats (e.g. Techstyles model 4600-350) and disposable Tyvek® sleeve covers (e.g. VWR Catalog # 10010-136)
Utensils for handling food samples:
Scalpel with disposable blades (if testing meats or similarly solid samples) Small disposable spoons (if testing liquid samples) Small disposable spatulas (if testing powdered or dry samples) Large-bore disposable transfer pipets (if testing homogenized samples)
Optional: PCR workstation/hood, e.g. CBS Scientific model P-030-202

Overview of the Agilent Porcine Detection Kit and Protocol

The Agilent Porcine Detection Kit contains reagents and materials for the extraction and isolation of DNA from food samples and other materials, as well as the subsequent QPCR amplification and detection of any porcine DNA that may be present in those samples. Because DNA isolated from processed food samples may contain contaminants that are inhibitory to PCR, the porcine detection QPCR kit is designed to amplify and detect two distinct targets in a single QPCR reaction: a porcine-specific DNA sequence and an external DNA control that enables you to detect inhibition of PCR.

The Agilent DNA Isolation Module

The DNA Isolation Kit is used to isolate DNA from food samples and other products of interest. The samples are first treated with proteinase K to release the nucleic acids into solution. DNA is then isolated by suspending the sample in binding buffer and loading onto a micro-spin cup containing a silica-based fiber matrix. The nucleic acids in the sample bind to the fiber matrix. The immobilized nucleic acid is then washed to remove contaminants and recovered in elution buffer. The eluted DNA is then ready for QPCR amplification.

The Agilent Porcine Detection QPCR Kit

The porcine detection assay mix contains PCR primers, fluorogenic probes and the external DNA control template (“Alien” DNA). The porcine-specific probe and primers amplify and detect a porcine-specific DNA sequence that is part of a very abundant gene (mitochondrial cytochrome b). The Alien control probe and primers amplify and detect a plasmid-encoded synthetic sequence that has no significant homology to any known nucleic acids. The porcine detection assay mix is supplied as a 10× mixture of components in a ratio for optimal performance.

The 2× porcine detection QPCR master mix supplies the remaining reagents necessary to simultaneously amplify the porcine-specific DNA target that may be present in the test sample and the Alien target

provided in the porcine detection assay mix. The master mix has been optimized for maximum performance on Agilent's Mx3000P and Mx3005P QPCR systems. Excellent results have also been obtained using the ABI 7500 Fast real-time PCR system, the BioRad CFX-96 system and the Qiagen Rotor-Gene Q real-time PCR instrument.

The porcine DNA control contains purified porcine DNA and is supplied for use as a positive control in the QPCR reactions.

Preprotocol Considerations

Avoiding Cross-Contamination

Take food samples that are to be tested straight to the laboratory to be weighed in labeled containers and then either processed to extract and isolate nucleic acid or stored for later extraction.

DNA isolation can be followed immediately by QPCR amplification (preferred method), or the DNA can be stored at 4°C for up to one month, or at –20°C or –80°C for longer periods.

CAUTION Handle samples with care! The porcine-specific target (when present) is a high copy number sequence. Mishandling can lead to contamination of other samples and cause false-positive results for porcine DNA detection. Read through the suggestions below to avoid cross-contamination of samples.

If possible, carry out each of the following steps of the protocol in separate rooms:

- Sample weighing and labeling
- DNA extraction and isolation
- QPCR reaction set up

These three work areas need to be supplied with the items necessary to carry out the tasks for that step. Avoid moving any items between the three areas, including marking pens, pipettors, etc. Items exposed to aerosols generated during sample homogenization should never be used in the other two workstations.

If you lack the lab space for three separate rooms, employ the following strategy. Establish separate work areas for sample weighing/labeling and QPCR reaction set up that are at opposite ends of the lab, and take sufficient care to prevent distribution of potential aerosols between the two workstations. Preferably, the area for QPCR reaction set up would be in a PCR hood. Establish a third work area for DNA extraction/isolation

that is in an entirely separate room from the other two areas. The extraction procedure is the step that is most likely to generate aerosols that can lead to cross-contamination.

For more information on setting up an optimal work area for PCR, see the following reference:
Dieffenbach, C.W. and Dveksler, G.S. (1993). Setting up a PCR laboratory. *Genome Research* 3:S2–S7.
(available at http://genome.cshlp.org/content/3/2/S2.refs.html)

2 Procedures

This chapter contains instructions on how to extract and isolate DNA from test samples and perform QPCR amplification with the isolated DNA. It also provides guidelines on analysis of the QPCR results to determine if porcine DNA is present in the test samples.

Preparations

To prepare the reagents

High Salt Wash Buffer

Prepare 1× High Salt Wash Buffer by adding 16 ml of 100% ethanol to the bottle of High-Salt Wash Buffer.

After adding the ethanol, affix the “EtOH Added” sticker (provided) to the cap. Close the cap of the container tightly and store at room temperature.

80% Ethanol

Prepare 80% ethanol by diluting 100% ethanol with DNase-free water.

To prepare 40 ml of 80% ethanol (enough for approximately 25 samples), add 8 ml of DNase-free water to 32 ml of 100% ethanol in a 50-ml conical polypropylene tube or a sterile glass bottle.

To prepare the test samples

Prior to DNA extraction and isolation, test samples must be weighed and either sliced or homogenized. The method for homogenization will depend on the nature of the sample, the volume of sample to be homogenized and the sampling procedure chosen.

NOTE

Take care when selecting a sample of the material for testing. In the food processing industry, sampling is a statistical process aimed at ensuring that the sample subjected to testing is representative of the product as a whole. Choose a sampling strategy that suits the material being tested. Several books and other resources are available that discuss sampling in detail (e.g. *Statistical Methods for Food Science: Introductory Procedures for the Food Practitioner* by John Bower; ISBN 978-4051-6764-2).

Suggested Homogenization Methods

- If a large volume is to be homogenized – e.g. a can of soup or multiple slices of meat – place the sample in a beaker (add a sufficient volume of sterile water if the sample is a solid) and use an immersion blender to homogenize. We recommend the KitchenAid model KHB100ER immersion blender and TriPour 400-ml disposable beakers (VWR Part # 25384-156). An aliquot of approximately 200 mg of homogenate can be used in the DNA extraction and isolation protocol.

  Do not re-use disposable beakers. For instructions on cleaning the immersion blender between samples, see *Chapter 4*, “Appendix”.

- If a small amount of meat is being tested, slice up a piece of the meat weighing between 25 and 200 mg and place the slices into a 1.5-ml microcentrifuge tube. DNA can be isolated directly from the slices, or the slices can be macerated using a disposable pellet pestle with an attachable spinning motor. We recommend the Kontes pellet pestle (Kontes Part # K749521-1590) and Kontes cordless motor (Kontes Part # K749540-0000).

  Always dispose of the pestle after one use.

NOTE If DNA extraction cannot be performed the same day as sample preparation, the samples may be frozen and stored at –20°C.

DNA Extraction and Isolation

To extract DNA

1. Pre-warm the Proteinase K Digestion Buffer to 65°C for 5 minutes in an incubator or water bath; invert to mix.
2. Prepare a working solution of Proteinase K by combining 200 µl of Proteinase K Digestion Buffer and 20 µl of Proteinase K per sample. Invert repeatedly to mix or gently mix on a vortex.

   Prepare a fresh working solution of Proteinase K before each use.
3. Add 220 µl of the Proteinase K working solution to each 1.5-ml tube of sliced, macerated or homogenized sample. Incubate the tubes at 65°C for 1 hour in an incubator or water bath.
4. Spin the tubes in a microcentrifuge for 3–5 minutes at maximum speed to pellet any undigested tissues.
5. Transfer 150 µl of each supernatant into a fresh 1.5-ml tube. Avoid transferring any undigested material from the bottom of the tube or any oily material that may be present at the top of the tube. This supernatant contains the DNA.

To isolate DNA

Before beginning, dispense 100 µl of Elution Buffer per sample (plus one sample excess) into a microcentrifuge tube and place the tube in an incubator set to 65°C.

1. Add 500 µl of Nucleic Acid Binding Buffer to each tube of supernatant. Vortex or pipette repeatedly until the solutions are mixed.
2. Transfer all 650 µl to a separate DNA Binding Spin Cup that has been seated within a 2-ml receptacle tube (provided) and snap the cap of the tube onto the top of the spin cup.
3. Spin the samples in a microcentrifuge for 1 minute at maximum speed to load the DNA onto the spin cup matrix.

4 Remove and retain the spin cups and discard the filtrates. For each sample, replace the spin cup in the receptacle tube, then add 500 µl of 1× High Salt Wash Buffer and cap the tube.

5 Spin the samples in a microcentrifuge at approximately 75% of maximum speed for 1 minute.

6 Remove and retain the spin cups and discard the filtrates. For each sample, replace the spin cup in the receptacle tube, then add 500 µl of 80% ethanol and cap the tube.

7 Spin the samples in a microcentrifuge at approximately 75% of maximum speed for 1 minute.

8 Repeat step 6 and step 7 two more times for a total of 3 washes with 500 µl of 80% ethanol.

9 After the third wash in 80% ethanol, remove and retain the spin cups and discard the filtrates. Replace the spin cups in their receptacle tubes and spin in a microcentrifuge for 2 minutes at maximum speed to dry the fiber matrix.

10 Transfer the spin cups to fresh 1.5-m1 collection tubes. Add 100 µl of Elution Buffer (pre-warmed to 65°C) to each spin cup directly on the fiber matrix inside the cup. Snap the caps of the collection tubes onto the spin cups and incubate at room temperature for 1 minute.

11 Spin the samples in a microcentrifuge at maximum speed for 1 minute.

12 The DNA is in the Elution Buffer in the microcentrifuge tube. Discard the spin cups and cap the tubes. The sample extracts may be stored at 4°C for up to one month. For long-term storage, keep extracts at –20°C or –80°C.

QPCR Amplification

NOTE

Please read the following notes carefully before beginning the QPCR set up:

Once the QPCR reagents are thawed (the 2× master mix, the porcine detection assay mix, the porcine DNA control and the sample extracts), keep the tubes on ice while setting up the reactions. See "Storage Conditions" on page 8 for recommendations on how to store the QPCR reagents after the initial thawing.

It is important to set up no-template control (NTC) reactions to screen for contamination of reagents and to provide a baseline for Ct determination of the Alien target.

A porcine DNA control sample is supplied for use in positive control reactions. Dilute this DNA 1:1000 (1 µl of supplied porcine DNA control + 999 µl of TE buffer or water). Perform the dilution step in a location away from the PCR workstation. **Set up the positive control reactions last, and be sure the test reactions and NTC reactions have been sealed before opening the tube of porcine DNA.**

We recommend using three volumes (0.5 µl, 2µl and 10 µl) for each sample extract being tested and setting up each volume in duplicate.

Have the cycler programmed and warmed up before you begin setting up the reactions.

The porcine detection assay mix contains fluorogenic probes; keep all solutions containing the assay mix protected from light.

To set up the QPCR reactions

1 Before you get started, first determine the number of reactions you will be running. In addition to the test reactions, include a positive control reaction and a no-template control (NTC) reaction, both of which should be run in duplicate.

Example: If 10 sample extracts are to be tested, set up test reactions with 0.5 µl, 2 µl and 10 µl of extract in duplicate (60 reactions), and include duplicate positive control reactions and duplicate NTC reactions (4 more reactions) for a total of 64 reactions.

2 Prepare the reactions by combining the components in Table 1. Prepare a single reagent mixture for all reactions that will be run simultaneously by scaling up the volumes listed in the table. Prepare enough reagent mixture for all your reactions plus *at least* one reaction volume excess.

Example: If you will be running 64 reactions, prepare enough reagent mixture for at least 65 reactions (and preferably more).

Table 1 PCR Reagent Mixture

Component	Volume per Reaction
2× QPCR Master Mix	12.5 µl
Porcine Detection Assay Mix	2.5 µl
Total volume	15 µl

3 Vortex the reagent mixture well, then distribute 15 µl to each individual well or PCR reaction tube.

4 Add the appropriate volume of nuclease-free water to each reaction so that the total reaction volume will be 25 µl once the DNA template is added. Appropriate water volumes based on reaction type are listed in Table 2.

Table 2 Volume of Water to Add

Reaction	Volume of Water
0.5 µl of sample extract	9.5 µl of water
2 µl of sample extract	8 µl of water
10 µl of sample extract	add no water
NTC reaction	10 µl of water
Positive control reaction	5 µl of water

5 Seal the NTC reactions by capping the tubes or applying strip caps.

6 Add the sample extracts to the appropriate test reactions in the appropriate volume (0.5 µl, 2 µl or 10 µl).

7 Seal the test reactions by capping the tubes or applying strip caps.

8 Add 5 µl of the diluted porcine DNA to the positive control reactions.

9 Seal the positive control reactions by capping the tubes or applying strip caps.

NOTE You do not need to mix the reactions after adding DNA. Mixing can create bubbles, which interfere with fluorescence detection.

To run the QPCR protocol

1 If possible, briefly spin the reactions in a centrifuge.

2 Place the reactions in the thermal cycler and run the QPCR program shown in Table 3. Set the cycler to detect and report fluorescence for the FAM and Cy5 channels during the 60°C annealing/extension step of each cycle.

Table 3 PCR Cycling Protocol

Segment	Number of Cycles	Temperature	Duration
1	1	95°C	10 minutes
2	40	95°C	30 seconds
		60°C	1 minute

To interpret the QPCR results

Use the thermal cycler's software to view the amplification plots of the QPCR data. The FAM channel detects the fluorescence from the porcine-specific target while the Cy5 channel detects fluorescence from the external Alien control target.

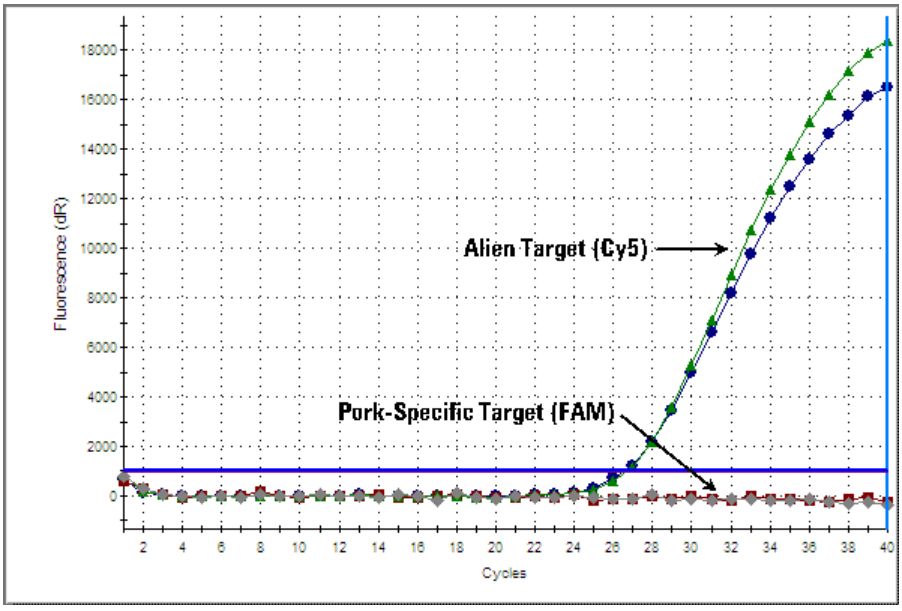
The images below show examples of data with guidelines on interpreting the results.

Samples that are negative for porcine DNA

In the NTC reactions and test reactions that do not contain porcine DNA, the amplification plots will look similar to those in Figure 1. Specifically, the porcine-specific target is not detected above threshold, but the Alien target is successfully amplified (indicating that PCR was not inhibited).

If the porcine-specific target is amplified in the NTC reactions, then your reactions have been contaminated. If the Alien target fails to amplify in any of the test reactions (or amplifies poorly), then your extracts may contain PCR inhibitors. See "Troubleshooting" on page 26 for suggestions.

Figure 1 Amplification plots from duplicate NTC reactions on the Agilent Mx3005P QPCR System. The Alien target (detected in the Cy5 channel) has a Ct near 26 in both reactions. The porcine-specific target (detected in the FAM channel) is not amplified.



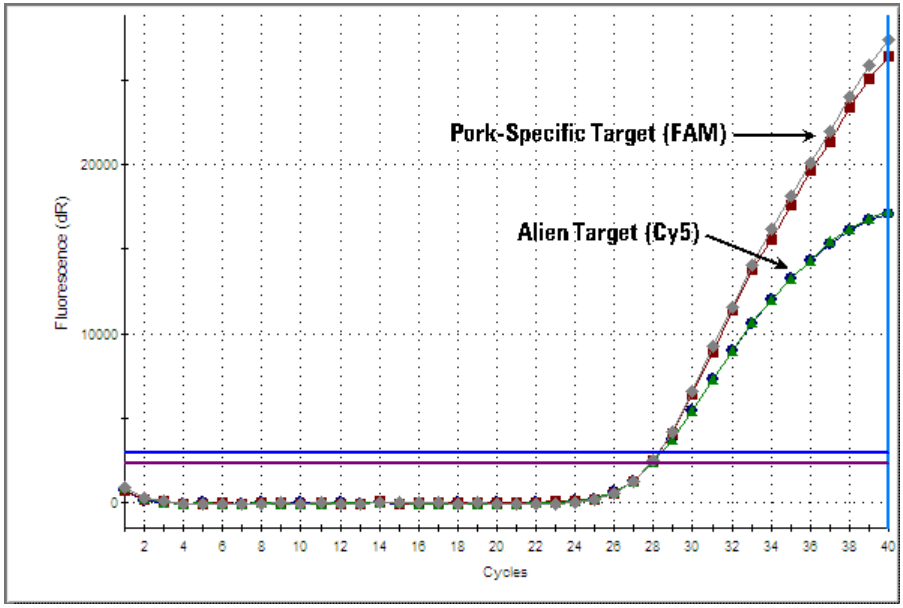
Samples that are positive for porcine DNA

In the positive control reactions and test reactions that contain porcine DNA, the amplification plots will look similar to those in Figure 2. Specifically, the porcine-specific target and the Alien target are both amplified and detectable above threshold.

The exact Ct of the porcine-specific target is dependent on the quantity of porcine DNA present in the sample extract (higher levels of porcine DNA result in lower Ct values). Testing three different volumes of each sample extract helps ensure that any porcine-positive extracts yield a detectable level of amplification in at least one of the tested volumes.

If the Alien target fails to amplify in any of the test reactions or in the positive control reactions, then the PCR failed. If you observe that the Alien target is amplified in the test reactions, but not as well as in the control reactions (i.e. the Ct is later in the test reactions), the sample extracts likely contain inhibitors of PCR that were carried over during the extraction and isolation procedures. See "Troubleshooting" on page 26 for suggestions.

Figure 2 Amplification plots from duplicate positive control reactions on the Agilent Mx3005P QPCR System. The Alien target is detected in the Cy5 channel. The porcine-specific target is detected in the FAM channel. The duplicate reactions behave similarly for both targets.



3
Troubleshooting Guide

This chapter offers recommendations for dealing with potential issues that may arise when using the Agilent Porcine Detection kit.

Troubleshooting

If the porcine-specific target is amplified in the NTC reactions

The reactions are contaminated.

✔ Repeat the experiment using a fresh set of QPCR reagents.

If the porcine-specific target fails to amplify in the positive control reactions

The PCR failed.

✔ Repeat the experiment, making sure to add the porcine DNA control in the proper concentration.

✔ Verify that the correct QPCR cycling protocol was used, including the 10-minute incubation at 95°C in segment 1. This 10-minute incubation is necessary to activate the *Taq* DNA Polymerase in the QPCR master mix.

If the Alien target fails to amplify in the test reactions

The PCR failed.

✔ Ensure that the recommended volume of extract (0.5, 2 or 10 µl) was added to each reaction.

✔ Verify that the correct QPCR cycling protocol was used, including the 10-minute incubation at 95°C in segment 1. This 10-minute incubation is necessary to activate the *Taq* DNA Polymerase in the QPCR master mix.

✔ Note that the Agilent DNA Isolation module is not effective on some types of samples, such as many cosmetics and other particularly waxy compounds.

Something in the sample extracts is inhibiting PCR in the test reactions.

✔ Follow recommendations for "If the Alien target is amplified poorly in the test reactions compared to the control reactions".

If the Alien target is amplified poorly in the test reactions compared to the control reactions (i.e. more than 2 cycles later)

Something in the sample extracts is inhibiting PCR in the test reactions.

✔ Try diluting the sample extracts by 10–100 fold and then re-run the QPCR. Diluting the extracts dilutes any PCR inhibitors present in the extracts and can improve amplification.

✔ If diluting the sample extracts does not reduce PCR inhibition, consider re-isolating the DNA from a fresh sample.

✔ If re-isolating DNA fails to reduce PCR inhibition, try incubating the sample extract with an iminodiacetic acid chelating resin (e.g. Sigma part number C7901-25G) using the manufacturer's instructions.

3 Troubleshooting Guide

If the Alien target is amplified poorly in the test reactions compared to the control reactions (i.e. more than 2 cycles later)

4 Appendix

This chapter contains instructions on decontaminating the immersion blender used for sample homogenization to prevent cross-contamination between samples.

To decontaminate an immersion blender

If using an immersion blender for sample homogenization, decontaminate the blender after each use. The instructions provided below pertain specifically to the KitchenAid model KHB100ER blender, but may be adapted for other models.

1. Disconnect the shank and head assembly from the blender's motor/handle unit.
2. Soak the head and shank piece in a 10–20% solution of bleach so that all but the very top of the shank is submerged. Let soak for 15 minutes.

   Make the bleach solution fresh each day.
3. While the head and shank piece is soaking, carefully wipe down the motor/handle unit with a paper towel moistened with the 10–20% bleach solution. Let the unit sit for 10 minutes, then wipe down with a paper towel moistened with water.

   Avoid getting bleach solution or water into the motor housing.
4. When the head and shank piece is finished soaking, transfer it to a container of water so that all but the very top of the shank is submerged. Swirl it in the water for several seconds to rinse off as much of the bleach as possible.
5. Connect the hand and shank piece to the motor/handle unit. Submerge the head into a second container of fresh water and turn on the blender for several seconds to thoroughly rise the head.

CAUTION Removing all traces of bleach from the head and shank of the blender is critical. Carry-over of bleach could result in DNA degradation or PCR inhibition for subsequent samples.

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In This Book

This document describes how to use the Porcine Detection Kit to extract genomic DNA from a food sample or other product and test it for the presence of porcine DNA using QPCR analysis.

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