Agilent MassCode PCR Research Solution

cDNA Synthesis, PCR, and PCR Purification

Protocol

Version C, April 2012

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Notices

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In this Guide...

This document describes how to synthesize cDNA from a sample of nucleic acids, perform MassCode PCR with the cDNA and purify the MassCode tag labeled PCR products using the the MassCode cDNA Synthesis Kit and the MassCode PCR Reagent Kit.

If you have comments about this protocol, send an email to masscode.support@agilent.com.

1 Before You Begin

Consult the Agilent MassCode PCR Research Solution Quick Start Guide for an overview of the MassCode PCR application and a description of how the protocol provided in this document fits into the overall MassCode PCR workflow. Before starting an experiment, make sure you read and understand the information in this chapter and have the necessary equipment and reagents needed.

2 Procedures

This chapter provides instructions on synthesizing cDNA from a mixture of nucleic acids, setting up and running the PCR amplification of the MassCode targets, and purifying the MassCode PCR reactions to isolate the tagged products.

3 Quick Reference Protocols

This chapter contains quick reference protocols with streamlined instructions covering the cDNA synthesis, MassCode PCR amplification, and PCR purification procedures. For more detailed instructions, see Chapter 2, “Procedures”.

Contents

1 Before You Begin

Consult the *Agilent MassCode PCR Research Solution Quick Start Guide* for an overview of the MassCode PCR application and a description of how the protocol provided in this document fits into the overall MassCode PCR workflow.

Before starting an experiment, make sure you read and understand the information in this chapter and have the necessary equipment and reagents needed.

Overview

Agilent's MassCode PCR Research Solution is a high-throughput platform for the detection of target nucleic acids based on multiplex PCR and LC/MS detection. An overview of the MassCode PCR Research Solution is shown in Figure 1.

Purpose of the protocol

This document provides a protocol for performing the cDNA synthesis, PCR amplification and PCR purification steps of the MassCode PCR workflow (see Figure 1) using Agilent's MassCode cDNA Synthesis Kit, the PCR Reagent Kit, and a target panel of MassCode tagged primers.

Protocol procedure

The starting material for this protocol is a nucleic acid mixture extracted from a sample of cells. The nucleic acids (a mixture of RNA and DNA) are first reverse transcribed to convert the RNA into first-strand cDNA using random primers and MassCode reverse transcriptase.

The DNA/cDNA samples are then used as the template in a multiplex PCR reaction with a mix of primers labeled with the MassCode tags.

The labeled PCR products are then purified using a 96-well purification plate that contains a silica-based fiber matrix in each well. The PCR products are loaded onto the purification plate, washed, and then eluted into an autosampler plate that can go directly into the LC/MS. The purification procedure has been optimized to recover MassCode PCR products.

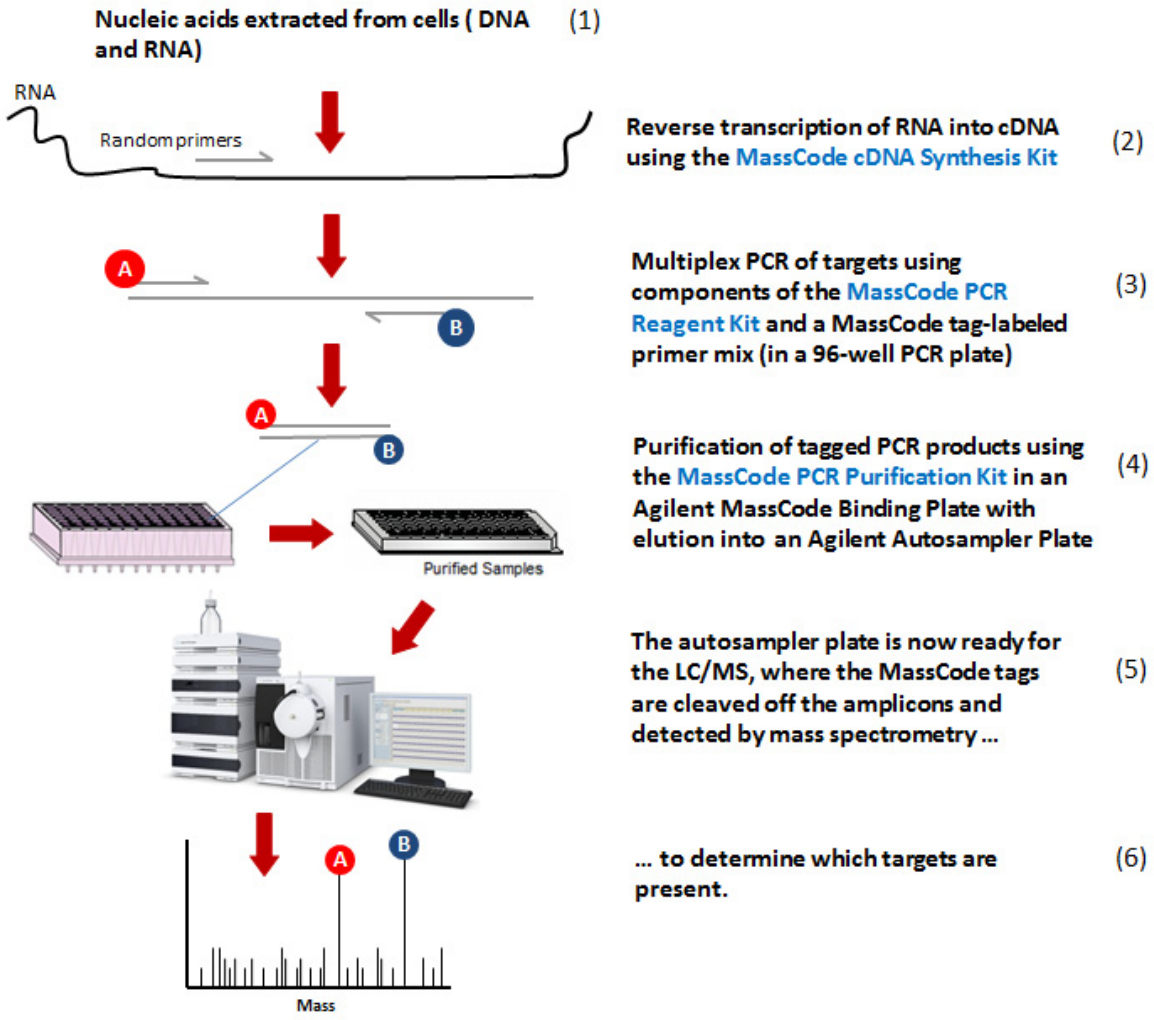

Figure 1 Overview of the MassCode PCR Research Solution and the path for sample processing

Important Information for Using the Protocol

Precautions to avoid RNase contamination

Ribonucleases, such as RNase A, are very stable enzymes responsible for RNA hydrolysis. RNase A can survive autoclaving and other standard methods of protein inactivation. Since RNases are present in the oils of the skin, wear gloves at all times when working with nucleic acid solutions that contain RNA. Use sterile tubes and micropipet tips handled with gloves only.

Contents of the kits

Table 1 Contents of the MassCode cDNA Synthesis Kit, Part Number 5190-3553

MassCode cDNA Synthesis Kit, 192 Reactions, Box #5190-3553	
MassCode Reverse Transcriptase	192 reactions
RNase Block	105 µl
10× MassCode RT Buffer	420 µl
dNTP	168 µl
Random Primers	660 µl
RNase-Free Water	2 × 1.5 ml

Table 2 Contents of the MassCode PCR Reagent Kit, Part Number 5190-3745

MassCode 2× PCR Master Mix Kit, 192 Reactions, Box #5190-3564	
MassCode 2× PCR Master Mix	192 reactions
RNase-Free Water	2 × 1.5 ml
MassCode PCR Purification Kit*, 192 Purifications, Box #5190-3605	
DNA Binding Solution	75 ml
PCR 5× Wash Buffer	50 ml
MassCode 96-well Binding Plates	2 plates
Adhesive Plate Sealer	15 sheets
96-well Waste Plates	2 plates
Autosampler 96-well Plate Covers	2 covers
Autosampler 96-well Plates	2 plates
MassCode System Suitability Calibrator, Box #5190-3743 (see Note below)	
20× System Suitability Calibrator	2 × 50 µl
MassCode Internal Amplification Control, 192 Reactions, Box #5190-3744	
MS2 Phage	200 µl
RNase-Free Water	2 × 1.5 ml

* You can purchase the PCR Purification Kit separately as a refill. See "Catalog information" on page 12.

NOTE Use the System Suitability Calibrator for the system suitability test that is performed on the LC/MS. Refer to the *LC/MS Prep and Maintenance Guide* for instructions on preparing the system suitability calibrator vial.

Table 3 MassCode Target Panels and Primers Available from Agilent

MassCode Respiratory Pathogen Panel Kit	Contact Agilent for ordering information
Custom MassCode Oligo, 10 nmol	Part Number 750010

Storage conditions

MassCode cDNA Synthesis Kit

Store all components at –20°C upon receipt.

MassCode PCR Reagent Kit

Store the following boxes at –20°C upon receipt:

- MassCode 2× PCR Master Mix Kit, Box #5190-3564

  After the initial thaw, you may store the 2× PCR Master Mix at 4°C for up to 6 months or continue storing at –20°C for long term storage.
- MassCode System Suitability Calibrator, Box #5190-3743
- MassCode Internal Amplification Control, Box #5190-3744

MassCode PCR Purification Kit

Store the PCR Purification Kit, Box #5190-3605, at room temperature.

Catalog information

The MassCode cDNA Synthesis Kit and MassCode PCR Reagent Kit are part of Agilent's MassCode PCR Research Solution. Catalog information for the reagent kits that are part of this solution is described in Table 4. To find part numbers for the available MassCode target panels, refer to Table 3.

Table 4 Reorder Part Numbers for MassCode Reagent Kits

Part Number	Description
5500-0111	MassCode Respiratory Sample Prep Kit
5190-3553	MassCode cDNA Synthesis Kit
5190-3745	MassCode PCR Reagent Kit
5190-3605	MassCode PCR Purification Kit (refill)

Required equipment and supplies

Table 5 Required Equipment and Supplies

Description
1.5-ml microcentrifuge tubes
Microcentrifuge
Agilent SureCycler 8800 with 96-well block (p/n G8800A and G8810A)
MassCode target panel or primers (see Table 3 for list)
PCR reaction tube strips and cap strips for cDNA synthesis reactions (optional), e.g. Agilent part number 410082 and 410086
Centrifuge for PCR reaction tube strips, if using
96-well PCR plates, non-skirted, and cap strips, e.g. Agilent part numbers 401333 and 410096
Centrifuge for 96-well plates
Multichannel pipettors (10 µl, 20 µl, 200 µl, and 1 ml volumes)
Disposable pipetting reservoirs, sterile, e.g. VWR part number 89094-676
Benchtop working rack for 96-well plates, e.g. Agilent part number 410094
96- well vacuum manifold with a deep well plate reservoir, e.g. Agilent part number 5185-5776
MS-grade, deionized water
100% ethanol

2 Procedures

This chapter provides instructions on synthesizing cDNA from a mixture of nucleic acids, setting up and running the PCR amplification of the MassCode targets, and purifying the MassCode PCR reactions to isolate the tagged products.

Synthesizing First-Strand cDNA

The cDNA synthesis step converts any RNA that is present in a nucleic acid mixture into first-strand cDNA.

Prepare for the calibrator reactions

In addition to setting up cDNA synthesis reactions with each of your nucleic acid test samples, set up three no-template calibrator reactions (CAL-NTC) and one internal amplification calibrator reaction (CAL-IACRNA). These reactions are described below.

The No-Template Calibrator Reactions (CAL-NTC)

In the CAL-NTC cDNA synthesis reactions, the nucleic acid sample is replaced with buffer (use the same buffer that your nucleic acid test samples are stored in). Since the reactions contain no nucleic acids, no PCR products are amplified during the subsequent PCR step.

The CAL-NTC reactions are used during LC/MS analysis to help set the cutoff values for target detection in the MassCode PCR software.

The Internal Amplification Calibrator Reaction (CAL-IACRNA)

The CAL-IACRNA cDNA synthesis reaction uses a nucleic acid sample prepared from MS2 phage alone (with no experimental sample added to the extraction reaction). The MS2 phage cDNA serves as the template for a phage-specific target during PCR. The abundance of this target is used during LC/MS analysis to help set the cutoff values for target detection in the MassCode PCR software. If you did not prepare an extraction reaction with MS2 phage alone, as instructed in the Respiratory Sample Preparation Kit Protocol, set up a cDNA synthesis reaction that includes whole MS2 phage particles (provided in the MassCode PCR Reagent Kit). The phage particles will lyse during the cDNA synthesis reaction and the genomic RNA will be accessible for reverse transcription.

Note that amplification of the phage-specific target also occurs in the test samples as an internal amplification control (IAC). The IAC verifies that the extraction, reverse transcription and PCR steps were successful for that sample.

Dilute the MS2 phage (if needed)

If your nucleic acid samples already contain MS2 phage RNA, proceed to "Anneal primers to template" on page 17. If you did not add MS2 phage to your samples prior to extraction, dilute the MS2 phage for addition into the cDNA synthesis reactions.

1 In a 1.5-ml microcentrifuge tube, combine 99 µl of RNase-free water with 1 µl of MS2 phage.

   MS2 phage and RNase-free water are provided in the PCR Reagent Kit (Box #5190-3744, MassCode Internal Amplification Control).

2 Mix the diluted phage well and keep on ice until needed.

   Do not store the diluted phage; prepare it fresh with each use.

   When setting up the primer annealing reactions for your test samples, add 2 µl of the diluted MS2 phage to each reaction.

NOTE Adding the MS2 phage to your experimental samples prior to nucleic acid extraction is preferable to adding it to the cDNA synthesis reactions. Adding the phage prior to extraction controls for possible nucleic acid loss that may occur during the extraction procedure.

Anneal primers to template

The primer annealing reactions are incubated in the SureCycler 8800. Set up the reactions in PCR reaction tube strips or on a PCR reaction plate.

1 Set up the primer annealing reaction tubes for the nucleic acid test samples.

   For each nucleic acid test sample, combine the components listed in Table 6. The table lists component volumes for reactions containing 4 µl or 10 µl of nucleic acid sample. A volume of 4 µl of nucleic acid sample is the minimum quantity we recommend. A volume of 10 µl of nucleic acid sample is optimal for maximizing the sensitivity of the assay.

Table 6 Primer Annealing Reagent Mixture for Test Sample Reactions

	Volume per reaction	
Component	**If adding 4 µl nucleic acid**	**If adding 10 µl nucleic acid**
Nucleic acid sample	4 µl	10 µl
Random primers	3 µl	3 µl
RNase-free water	8.7 µl*	2.7 µl*
Total volume	15.7 µl	15.7 µl

* If needed, add 2 µl of the diluted MS2 phage to each reaction and reduce the volume of water by 2 µl.

2 Set up the primer annealing reaction tubes for the three CAL-NTC reactions.

For each CAL-NTC reaction, combine the components listed in Table 7. The table lists component volumes for reactions containing 4 µl or 10 µl of nucleic acid storage buffer. The volume of storage buffer in the CAL-NTC reactions needs to be the same as the volume of nucleic acid used for the test sample reactions in step 1.

Table 7 Primer Annealing Reagent Mixture for the CAL-NTC Reactions

	Volume per reaction	
Component	**If adding 4 µl storage buffer**	**If adding 10 µl storage buffer**
Nucleic acid storage buffer	4 µl	10 µl
Random primers	3 µl	3 µl
RNase-free water	8.7 µl	2.7 µl
Total volume	15.7 µl	15.7 µl

3 Set up the primer annealing reaction tubes for the CAL-IACRNA reaction.

Combine the components listed in Table 8. The table lists component volumes for reactions containing MS2 phage nucleic acids or diluted MS2 phage (see "Dilute the MS2 phage (if needed)" on page 17).

Table 8 Primer Annealing Reagent Mixture for the CAL-IACRNA Reaction

Component	Volume per reaction	
	If using MS2 nucleic acids	If using whole MS2 phage
MS2 phage nucleic acid sample	4 µl	—
Diluted MS2 phage	—	2 µl
Random primers	3 µl	3 µl
RNase-free water	8.7 µl	10.7 µl
Total volume	15.7 µl	15.7 µl

4 Incubate the primer annealing reactions at 70°C for 5 minutes in the SureCycler 8800.

We recommend bringing the SureCycler 8800 up to temperature and then pausing the program before adding the reaction tubes.

5 Remove the reactions from the SureCycler 8800, spin them briefly in a microcentrifuge, and place them directly on ice.

Perform reverse transcription

1 Prepare the reverse transcription reagent mixture by combining the components listed in Table 9.

The volumes listed are sufficient for one reaction. Prepare enough reagent mixture for all your reactions plus *at least* one reaction volume excess. Keep all reactions and reagents on ice.

Table 9 Reverse Transcription Reagent Mixture

Component	Volume per reaction
10× MassCode RT Buffer	2 µl
dNTP Mix	0.8 µl
RNase Block	0.5 µl
MassCode Reverse Transcriptase	1 µl

2 Add 4.3 µl of the reverse transcription reagent mixture to each primer annealing reaction. Mix the reactions well (do not vortex).

3 Incubate the reactions in the SureCycler 8800 according to the program listed in Table 10.

Table 10 cDNA Synthesis Incubation Program

Incubation Step	Temperature	Duration
Primer Annealing	25°C	10 minutes
1st Strand Synthesis	42°C	60 minutes
Heat Inactivation of Reverse Transcriptase	70°C	15 minutes
Storage	4°C	Hold indefinitely

CAUTION Do not let the cDNA synthesis reactions incubate at 4°C overnight.

4 When the program is complete, remove the reactions from the SureCycler 8800.

You can now perform the MassCode PCR amplification protocol (see "Amplifying MassCode Targets by PCR" on page 21) or store the cDNA at –80°C for later use.

Amplifying MassCode Targets by PCR

The PCR amplification step amplifies target sequences using a multiplex primer mix. The primers in this mix are each labeled with a unique chemical moiety known as a MassCode tag that will later be used for target identification by mass spectrometry. The primer mix can be of a custom design or purchased as part of an Agilent MassCode target panel (see Table 3).

Design a PCR plate setup

The PCR is carried out in a 96-well PCR plate (not provided). Perform PCR on your test cDNA samples, the CAL-IACRNA cDNA sample and the three CAL-NTC cDNA samples. Additionally, set up PCR reactions for any positive template calibrator (PTC) samples that were included with your MassCode target panel. The respiratory panel includes 4 PTC samples; run duplicate PCR reactions for each PTC. Your plate needs a total of 12 calibrator reactions in order for the MassCode PCR software to properly analyze the data.

	1	2	3	4	5	6	7	8	9	10	11	12
A	CAL-NTC	CAL-NTC	CAL-PTC1	CAL-PTC2	CAL-PTC3	CAL-PTC4	CAL-IACRNA	CAL-NTC	CAL-PTC1	CAL-PTC2	CAL-PTC3	CAL-PTC4
B	Unknown reaction 1	Unknown reaction 2	Unknown reaction 3	Unknown reaction 4	Unknown reaction 5	Unknown reaction 6	Unknown reaction 7	Unknown reaction 8	Unknown reaction 19	Unknown reaction 10	Unknown reaction 11	Unknown reaction 12
C	Unknown reaction 13	Unknown reaction 14	Unknown reaction 15	Unknown reaction 16	Unknown reaction 17	Unknown reaction 18	Unknown reaction 19	Unknown reaction 20	Unknown reaction 21	Unknown reaction 22	Unknown reaction 23	Unknown reaction 24
D	Unknown reaction 25	Unknown reaction 26	Unknown reaction 27	Unknown reaction 28	Unknown reaction 29	Unknown reaction 30	Unknown reaction 31	Unknown reaction 32	Unknown reaction 33	Unknown reaction 34	Unknown reaction 35	Unknown reaction 36
E	Unknown reaction 37	Unknown reaction 38	Unknown reaction 39	Unknown reaction 40	Unknown reaction 41	Unknown reaction 42	Unknown reaction 43	Unknown reaction 44	Unknown reaction 45	Unknown reaction 46	Unknown reaction 47	Unknown reaction 48
F	Unknown reaction 49	Unknown reaction 50	Unknown reaction 51	Unknown reaction 52	Unknown reaction 53	Unknown reaction 54	Unknown reaction 55	Unknown reaction 56	Unknown reaction 57	Unknown reaction 58	Unknown reaction 59	Unknown reaction 60
G	Unknown reaction 61	Unknown reaction 62	Unknown reaction 63	Unknown reaction 64	Unknown reaction 65	Unknown reaction 66	Unknown reaction 67	Unknown reaction 68	Unknown reaction 69	Unknown reaction 70	Unknown reaction 71	Unknown reaction 72
H	Unknown reaction 73	Unknown reaction 74	Unknown reaction 75	Unknown reaction 76	Unknown reaction 77	Unknown reaction 78	Unknown reaction 79	Unknown reaction 80	Unknown reaction 81	Unknown reaction 82	Unknown reaction 83	Unknown reaction 84

Figure 2 An example of a plate setup for PCR

An example of a recommended plate setup is shown in Figure 2. The calibrator reactions are set up in row A of the plate. In this example, the target panel includes 4 CAL-PTC samples and each is set up in duplicate. The three CAL-NTC reactions are in wells A1, A2 and A8. The PCR reaction with the CAL-IACRNA sample is set up in a single well (A7). The test reactions are set up in rows B through H. With this setup, you can test up to 84 test samples in a single assay.

Prepare the PCR reactions

1 Prepare the PCR reagent mixture by combining the components in Table 11. Keep all reactions and reagents on ice.

The volumes listed are sufficient for one reaction. Prepare enough reagent mixture for *at least* one reaction volume excess.

Table 11 PCR Reagent Mixture

Component	Volume per reaction
RNase-free water	3.2 µl*
MassCode 2× PCR Master Mix	10 µl
MassCode primer mix	2.8 µl (250 nM each primer; see Note below)

* 3.2 µl of water is appropriate for PCR reactions containing 4 µl of cDNA. If you need to maximize the sensitivity of the assay, you can add as much as 7.2 µl of cDNA and reduce the volume of water accordingly.

NOTE

2.8 µl is the recommended volume for a 28-plex primer mix (e.g. the primer mix provided in the Respiratory Pathogen Panel Kit). If you are designing your own MassCode primers, maintain a concentration of 250 nM for each primer in the final PCR reactions. For primers with a stock concentration of 100 mM, you need to add 0.05 µl of each primer (or 0.1 µl of each primer set) per reaction.

Note that you may need to adjust the volume of water in the PCR reagent mixture to obtain a final volume of 20 µl in each PCR reaction.

2 Pipet the reagent mixture up and down to mix, then distribute 16 µl to each well on the PCR plate.

3 Add 4 µl of cDNA or PTC to each well.

See "Design a PCR plate setup" on page 21 for guidelines.

Run the PCR program

1 Place the PCR plate in the SureCycler 8800. If you are using the respiratory pathogen panel, run the program in Table 12. If you using a custom target panel, run a PCR program suitable for your primer mix.

Table 12 PCR Cycling Program

Segment	Number of Cycles	Temperature	Duration
1	1	95°C	3 minutes
2	10	95°C	20 seconds
		65–56°C (temperature increment of –1°C/cycle)	50 seconds
3	15	95°C	20 seconds
		55°C	50 seconds
4	10	95°C	20 seconds
		55°C	80 seconds
5	10	95°C	20 seconds
		55°C	2 minutes
6	1	72°C	5 minutes

2 Remove the plate from the SureCycler 8800.

You can now perform the PCR purification protocol (see "Purifying the PCR Products" on page 24) or store the plate at –20°C for later use.

NOTE The MassCode tags on the PCR products are light sensitive. Minimize their exposure to light.

Purifying the PCR Products

PCR purification removes excess primers, nucleotides, buffer components, and enzymes. It also removes any primer dimers that may have formed during cycling. Eliminating these excess reagents and primer dimers is important for reducing background signals in the mass spectrometry data.

The MassCode PCR Purification Kit is included as part of the PCR Reagent Kit, but you can purchase it separately as a refill (p/n 5190-3745).

Prepare the reagents

DNA Binding Solution (also used as the wash #1 solution)

1 Prepare 100 ml of 30% ethanol by combining 30 ml of 100% ethanol with 70 ml of MS-grade dH_20.

2 Mix 75 ml of the provided DNA Binding Solution with 75 ml of 30% ethanol.

Store the prepared DNA Binding Solution tightly sealed at room temperature.

PCR Wash Buffer (the wash #2 solution)

1 Add 200 ml of 100% ethanol to the stock bottle of 5× PCR Wash Buffer.

The addition of ethanol brings the PCR wash buffer to a 1× concentration.

2 Using a permanent marker, mark the “ADD ETHANOL BEFORE USE” check box printed on the bottle.

Store the 1× PCR Wash Buffer tightly sealed at room temperature.

80% Ethanol (the wash #3 solution)

- Mix 160 ml of 100% ethanol with 40 ml of MS-grade dH_20.

Store the 80% ethanol at room temperature.

Purify the PCR products

NOTE If you are not using all 96 wells of the binding plate, you can cover the unused portion of the plate with adhesive plate sealer and use those wells in a later experiment.

Immobilize the PCR products on the binding plate matrix

1 Add 180 µl of the prepared DNA Binding Solution (refer to "Prepare the reagents" on page 24) to each 20-µl PCR reaction on the PCR plate. Pipet up and down to mix.

2 Place a MassCode 96-well binding plate on a vacuum manifold that contains a deep well plate reservoir. Using a multichannel pipettor, transfer the PCR product/DNA Binding Solution mixtures into the wells of the binding plate. Perform the transfer in a way that maintains the original plate setup configuration.

3 Perform the vacuum/centrifugation procedure on the binding plate according to the table below:

Vacuum/Centrifugation Procedure for Immobilizing the PCR Products

Step	Details/Comments
a Apply 400 mbar of vacuum to the binding plate until each well is dry (approximately 1 minute) and then continue for 1 minute more.	• The vacuum force may decrease as the wells become dry.
b Release the vacuum and remove the binding plate from the manifold. Blot the bottom of the binding plate to remove excess liquid.	• To blot, tip the plate at an approximately 45° angle and touch just one corner of the plate to a paper towel.
c Place the binding plate on top of a 96-well waste plate (provided). Cover the top of the binding plate with adhesive plate sealer and then adhere the two plates together.	• To adhere the plates, wrap them together with a rubber band or tape them together along the edges.
d Spin the stacked plates in a room temperature centrifuge for 3 minutes at 1500 × *g*.	• If any liquid remains in the wells at the end of the spin, perform the spin again.
e Disassemble the stacked plates.	• The PCR products are retained in the matrix of the binding plate wells. • Set aside the waste plate. You will use it again during the wash procedure.

Wash the PCR products on the matrix

1 Remove the plate sealer from the binding plate and add 500 µl of the prepared DNA Binding Solution to each well.

This step is wash #1.

2 Perform the vacuum/centrifugation procedure on the binding plate according to the table below:

Vacuum/Centrifugation Procedure for Washing the PCR Products

Step	Details/Comments
a Apply 400 mbar of vacuum to the binding plate until each well is dry (approximately 1 minute) and then continue for 1 minute more.	• The vacuum force may decrease as the wells become dry.
b Release the vacuum and remove the binding plate from the manifold. Blot the bottom of the binding plate to remove excess liquid.	• To blot, tip the plate at an approximately 45° angle and touch just one corner of the plate to a paper towel.
c Place the binding plate on top of the waste plate. Cover the top of the binding plate with adhesive plate sealer and then adhere the two plates together.	• To adhere the plates, wrap them together with a rubber band or tape them together along the edges.
d Spin the stacked plates in a room temperature centrifuge at 1500 × *g*.	• For wash #1 and wash #2, spin for 3 minutes. • For wash #3, spin for 5 minutes.
e Open the vacuum manifold and discard the wash solution from the waste plate. Replace the waste plate back on the vacuum manifold	• This step can be performed while the plates are spinning in the centrifuge.
f Remove the stacked plates from the centrifuge and disassemble.	• The PCR products are retained in the matrix of the binding plate wells.

3 Remove the plate sealer from the binding plate and add 700 µl of **1× PCR wash buffer** (see "Prepare the reagents" on page 24) to each well.

This step is wash #2.

4 Repeat the vacuum/centrifugation procedure for washing the PCR products outlined in step 2 above.

5 Remove the plate sealer from the binding plate and add 700 µl of **80% ethanol** (see "Prepare the reagents" on page 24) to each well.

This step is wash #3.

6 Repeat the vacuum/centrifugation procedure for washing the PCR products outlined in step 2 above.

Elute the PCR products into the LC/MS autosampler plate

1 Place the binding plate on top of a 96-well plate that is suitable for use in the LC/MS autosampler (provided in the MassCode PCR Purification Kit; p/n 5190-3728). Check that the two plates are oriented in the same direction (i.e. well A1 of the binding plate is sitting on top of well A1 of the autosampler plate).

2 Remove the plate sealer from the binding plate and add 70 µl of MS-grade dH_20 directly on top of the fiber matrix of each well.

If you want to run the plate in the LC/MS twice, increase the volume of MS-grade dH_20 to 140 µl. Note that eluting in a higher volume decreases sensitivity of the assay.

3 Replace the plate sealer on the binding plate. Tape the two plates together along the edges or wrap them together with a rubber band. Allow the plates to incubate at room temperature for 2 minutes.

4 Spin the stacked plates in a room temperature centrifuge at 1500 × *g* for 5 minutes.

5 Remove the plates from the centrifuge and cover the autosampler plate with an autosampler plate cover (provided).

6 Place the plate in the autosampler (instructions below) to begin the LC/MS analysis or store it at 4°C (for up to 1 week) or –20°C (for long term storage).

Place the plate in the autosampler

- Place the autosampler plate in the front position of the LC/MS autosampler (2 positions for plates: one in front and one in back).
- Place the plate so that row A is in the back and row H faces the front.
- If you want to turn off the light inside the autosampler, see the *Online Help* of the MassCode PCR program. (The default setting is On.)

You are now ready to set up the plate and run parameters with the MassCode PCR program.

3 Quick Reference Protocols

This chapter contains quick reference protocols with streamlined instructions covering the cDNA synthesis, MassCode PCR amplification, and PCR purification procedures. For more detailed instructions, see Chapter 2, “Procedures”.

Synthesize first-strand cDNA

1 If you will be adding MS2 phage to the cDNA synthesis reactions, prepare a 1:100 dilution of the provided stock.

   a In 1.5-ml microcentrifuge tube, combine 99 µl of RNase-free water with 1 µl of MS2 phage.

   b Mix the diluted phage well and keep on ice until needed.

2 Set up the primer annealing reactions in PCR reaction tubes for the nucleic acid test samples, the three CAL-NTC reactions, and the CAL-IACRNA reaction.

   • For each test sample reaction, combine the components in Table 13.

Table 13 Primer Annealing Reagent Mixture for Test Sample Reactions

	Volume per reaction	
Component	**If adding 4 µl nucleic acid**	**If adding 10 µl nucleic acid**
Nucleic acid sample	4 µl	10 µl
Random primers	3 µl	3 µl
RNase-free water	8.7 µl*	2.7 µl*
Total volume	15.7 µl	15.7 µl

* If needed, add 2 µl of the diluted MS2 phage to each reaction and reduce the volume of water by 2 µl.

   • For each CAL-NTC reaction, combine the components in Table 14.

Table 14 Primer Annealing Reagent Mixture for the CAL-NTC Reactions

	Volume per reaction	
Component	**If adding 4 µl storage buffer**	**If adding 10 µl storage buffer**
Nucleic acid storage buffer	4 µl	10 µl
Random primers	3 µl	3 µl
RNase-free water	8.7 µl	2.7 µl
Total volume	15.7 µl	15.7 µl

- For the CAL-IACRNA reaction, combine the components in Table 15.

Table 15 Primer Annealing Reagent Mixture for the CAL-IACRNA Reaction

	Volume per reaction	
Component	**If using MS2 nucleic acids**	**If using whole MS2 phage**
MS2 phage nucleic acid sample	4 µl	—
Diluted MS2 phage	—	2 µl
Random primers	3 µl	3 µl
RNase-free water	8.7 µl	10.7 µl
Total volume	15.7 µl	15.7 µl

3 Incubate the reactions at 70°C for 5 minutes in the SureCycler 8800.

4 Remove the reactions from the cycler, spin them briefly in a microcentrifuge and place directly on ice.

5 Prepare the reverse transcription reagent mixture by combining the components listed in Table 16. Keep all reactions and reagents on ice.

Table 16 Reverse Transcription Reagent Mixture

Component	Volume per reaction
10× MassCode RT Buffer	2 µl
dNTP Mix	0.8 µl
RNase Block	0.5 µl
MassCode Reverse Transcriptase	1 µl

6 Add 4.3 µl of the reverse transcription reagent mixture to each primer annealing reaction. Mix the reactions well (do not vortex).

7 Incubate the reactions in the SureCycler 8800 using the program listed in Table 17.

Table 17 cDNA Synthesis Incubation Program

Incubation Step	Temperature	Duration
Primer Annealing	25°C	10 minutes
1st Strand Synthesis	42°C	60 minutes
Heat Inactivation of Reverse Transcriptase	70°C	15 minutes
Storage	4°C	Hold indefinitely

8 When the program is complete, promptly remove the reactions from the cycler. You can now amplify MassCode targets by PCR or store the cDNA at –80°C for later use.

Amplify MassCode targets by PCR

1 Prepare the PCR reagent mixture by combining the components in Table 18. Prepare enough reagent mixture for all reactions plus *at least* one reaction volume excess.

Table 18 PCR Reagent Mixture

Component	Volume per reaction
RNase-free water	3.2 µl*
MassCode 2× PCR Master Mix	10 µl
Target Panel Primer Mix	2.8 µl**

* 3.2 µl of water is appropriate for PCR reactions containing 4 µl of cDNA.

**2.8 µl of primer mix per reaction is appropriate for the respiratory pathogen panel.

2 Vortex the mixture, then distribute 16 µl to each well on the PCR plate.

3 Add 4 µl of cDNA or PTC to each well. See "Design a PCR plate setup" on page 21 for guidelines.

4 Place the plate in the SureCycler 8800. For the respiratory target panel, run the program in Table 19. For a custom target panel, run a program suitable for your primer mix.

Table 19 PCR Cycling Program

Segment	Number of Cycles	Temperature	Duration
1	1	95°C	3 minutes
2	10	95°C	20 seconds
		65–56°C (temperature increment of –1°C/cycle)	50 seconds
3	15	95°C	20 seconds
		55°C	50 seconds
4	10	95°C	20 seconds
		55°C	80 seconds
5	10	95°C	20 seconds
		55°C	2 minutes
6	1	72°C	5 minutes

5 Remove the plate from the SureCycler 8800. You can now purify the PCR products or store the plate at –20°C for later use.

Purify the PCR products

1 Add 180 µl of the prepared DNA Binding Solution (refer to "Prepare the reagents" on page 24) to each 20-µl PCR reaction on the PCR plate. Pipet up and down to mix.

2 Place a MassCode 96-well binding plate on a vacuum manifold that contains a deep well plate reservoir. Using a multichannel pipettor, transfer the PCR product/DNA Binding Solution mixtures into the wells of the binding plate. Perform the transfer in a way that maintains the original plate setup configuration.

3 Perform the vacuum/centrifugation procedure on the binding plate according to the table below:

Vacuum/Centrifugation Procedure for Immobilizing the PCR Products

Step	Details/Comments
a Apply 400 mbar of vacuum to the binding plate until each well is dry (approximately 1 minute) and then continue for 1 minute more.	• The vacuum force may decrease as the wells become dry.
b Release the vacuum and remove the binding plate from the manifold. Blot the bottom of the binding plate to remove excess liquid.	• To blot, tip the plate at an approximately 45° angle and touch just one corner of the plate to a paper towel.
c Place the binding plate on top of a 96-well waste plate (provided). Cover the top of the binding plate with adhesive plate sealer and then adhere the two plates together.	• To adhere the plates, wrap them together with a rubber band or tape them together along the edges.
d Spin the stacked plates in a room temperature centrifuge for 3 minutes at 1500 × *g*.	• If any liquid remains in the wells at the end of the spin, perform the spin again.
e Disassemble the stacked plates.	• The PCR products are retained in the matrix of the binding plate wells. • Set aside the waste plate. You will use it again during the wash procedure.

4 Remove the plate sealer from the binding plate and add 500 µl of the prepared DNA Binding Solution (refer to "Prepare the reagents" on page 24) to each well.

This step is wash #1.

5 Perform the vacuum/centrifugation procedure on the binding plate according to the table below:

Vacuum/Centrifugation Procedure for Washing the PCR Products

Step	Details/Comments
a Apply 400 mbar of vacuum to the binding plate until each well is dry (approximately 1 minute) and then continue for 1 minute more.	• The vacuum force may decrease as the wells become dry.
b Release the vacuum and remove the binding plate from the manifold. Blot the bottom of the binding plate to remove excess liquid.	• To blot, tip the plate at an approximately 45° angle and touch just one corner of the plate to a paper towel.
c Place the binding plate on top of the waste plate. Cover the top of the binding plate with adhesive plate sealer and then adhere the two plates together.	• To adhere the plates, wrap them together with a rubber band or tape them together along the edges.
d Spin the stacked plates in a room temperature centrifuge at 1500 × *g*.	• For wash #1 and wash #2, spin for 3 minutes. • For wash #3, spin for 5 minutes.
e Open the vacuum manifold and discard the wash solution from the waste plate. Replace the waste plate back on the vacuum manifold	• This step can be performed while the plates are spinning in the centrifuge.
f Remove the stacked plates from the centrifuge and disassemble.	• The PCR products are retained in the matrix of the binding plate wells.

6 Remove the plate sealer from the binding plate and add 700 µl of **1× PCR wash buffer** (see "Prepare the reagents" on page 24) to each well.

This step is wash #2.

7 Repeat the vacuum/centrifugation procedure for washing the PCR products outlined in step 5 above.

8 Remove the plate sealer from the binding plate and add 700 µl of **80% ethanol** (see "Prepare the reagents" on page 24) to each well.

This step is wash #3.

9 Repeat the vacuum/centrifugation procedure for washing the PCR products outlined in step 5 above.

10 Place the binding plate on top of a 96-well autosampler plate (provided). Check that the two plates are oriented in the same direction.

11 Remove the plate sealer from the binding plate and add 70 µl of MS-grade dH_20 directly on top of the fiber matrix of each well.

If you want to run the plate in the LC/MS twice, increase the volume of MS-grade dH_20 to 140 µl (note that this decreases sensitivity of the assay).

12 Replace the plate sealer on the binding plate. Tape the two plates together along the edges or wrap them together with a rubber band. Allow the plates to incubate at room temperature for 2 minutes.

13 Spin the stacked plates in a room temperature centrifuge at 1500 × *g* for 5 minutes.

14 Remove the plates from the centrifuge and cover the autosampler plate with an autosampler plate cover (provided).

The purified PCR products are in the bottom of each well of the autosampler plate. You can now place the plate in the LC/MS autosampler for mass spectrometry analysis or store the covered plate at 4°C (for up to 1 week) or –20°C (for long term storage).

15 Place the covered autosampler plate in the LC/MS autosampler or store it at 4°C (for up to 1 week) or –20°C (for long term storage).

- To place the plate in the autosampler, place it in the front position of the autosampler so that row A is in the back and row H faces the front.
- To store the plate, place it at 4°C (for up to 1 week) or –20°C (for long term storage).

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In This Book

This document describes how to synthesize cDNA from a sample of nucleic acids, perform MassCode PCR with the cDNA and purify the MassCode tag labeled PCR products using the the MassCode cDNA Synthesis Kit and the MassCode PCR Reagent Kit.

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