SureDirect Blood PCR Kit

Catalog #600920 (100 Reactions) and Catalog #600930 (500 Reactions)

Protocol

Version C.0, June 2015

For Research Use Only. Not for use in diagnostic procedures.

Agilent Technologies

Notices

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CAUTION

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A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

In this Guide...

This guide describes an optimized protocol for using the SureDirect Blood PCR Kit to amplify targets of interest from blood or blood-derivative samples.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 Analysis of Liquid Blood Samples

This chapter describes the protocol steps for PCR analysis of targets of interest directly from liquid blood samples.

3 Analysis of Dried Blood Samples

This chapter describes the protocol steps for PCR analysis of targets of interest directly from blood samples dried on a solid support.

What's New in Version C.0

- Updated product labeling statement

Content

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.

Procedural Notes

- Follow your institution's guidelines for safety procedures and other practices required for working with blood samples.
- In general, follow Biosafety Level 1 (BL1) safety rules.
- Ensure that reagent mixtures are thoroughly mixed, by pipetting up-and-down or by gentle vortexing, before distributing to the samples.

Safety Notes

CAUTION

- Wear appropriate personal protective equipment (PPE) when working in the laboratory.

Reagents Required for the Protocol

Table 1 Required Reagents for the SureDirect Blood PCR Kit protocol

Description	Vendor and part number
SureDirect Blood PCR Kit* 100 Reactions 500 Reactions	Agilent p/n 600920 p/n 600930
Nuclease-free Water (PCR-grade)	Cromasolv water, Sigma, p/n 27073, or equivalent
PCR Primer Pair, desalted	IDT, or equivalent

* See Table 2, below, for a list of included reagents.

Contents of the SureDirect Blood PCR Kit

Table 2 SureDirect Blood PCR Kit Contents

Reagent	Format
SureDirect Blood PCR 2× MM (Master Mix provided at 2× concentration)	tube with red cap
DMSO	tube with green cap
10× Control Primers + Template	tube with clear cap

Required Equipment for Analysis of Liquid Blood Samples

Table 3 Equipment required for analysis of liquid blood samples

Description	Vendor and part number
Thermal Cycler	Agilent SureCycler 8800, p/n G8800A or equivalent
8-well PCR strip tubes	Agilent, p/n 410092
Tube cap strips (8 domed caps per strip)	Agilent, p/n 410096
Centrifuge with strip tube-compatible rotor, such as Eppendorf 5424 benchtop microcentrifuge with strip-tube rotor	VWR p/n 93000-196 (centrifuge) and p/n 80094-150 (rotor), or equivalent
P20 and P200 pipettes	Pipetman or equivalent
Ice bucket	General laboratory supplier
Vortex mixer	General laboratory supplier

Required Equipment for Analysis of Dried Blood Samples

Table 4 Equipment required for analysis of dried blood samples

Description	Vendor and part number
Whatman 903 Protein Saver Cards	GE Healthcare, p/n 10534320
Sample disc puncher with 2-mm ID bore	GE Healthcare, p/n WB100007 or equivalent
Sterile forceps	General laboratory supplier
Thermal Cycler	Agilent SureCycler 8800, p/n G8800A or equivalent
8-well PCR strip tubes	Agilent, p/n 410092
Tube cap strips (8 domed caps per strip)	Agilent, p/n 410096
P20 and P200 pipettes	Pipetman or equivalent
Ice bucket	General laboratory supplier
Vortex mixer	General laboratory supplier
Microcentrifuge	General laboratory supplier

2
Analysis of Liquid Blood Samples

For PCR analysis of blood samples in liquid form using the SureDirect Blood PCR Kit, follow the instructions in this chapter.

For analysis of dried blood samples, instead see Chapter 3 on page 23.

PCR primer considerations

Design PCR primers for analysis of blood samples using the following considerations:

- The SureDirect Blood PCR Kit can be used to amplify a variety of target sizes and composition profiles with some optimization of conditions. For assays that require the least optimization, however, design targets in the 200 to 400 bp range and with typical GC-content. The positive control provided with the kit produces a 248-bp amplicon.
- Primers should have a calculated T_m value of approximately 63°C to 73°C. You can use the IDT OligoAnalyzer 3.1 tool, provided by the recommended primer vendor, to estimate primer T_m. The recommended annealing temperature for amplification using the SureDirect Blood PCR Kit is 5°C lower than the average primer T_m.

Before using the chosen primer pair for target amplification from blood samples, validate the specificity and yield of the PCR reaction using purified genomic DNA as template.

Blood sample properties and sample quantity considerations

- Blood samples to be analyzed with the SureDirect Blood PCR Kit in liquid form should be obtained from a standard blood collection tube containing an anticoagulant such as heparin, EDTA, or citrate. Blood collected in the presence of an anticoagulant may be stored at –80°C for later analysis, if required. Blood samples stored at –80°C should be divided into single-use aliquots and should be thawed only once.
- Protocols are provided for a typical, optimized PCR assay, using 5-µL human blood samples present at 20% (v/v) of the complete PCR reaction. You can design different assay conditions, using modified blood sample volume and proportion of blood in the PCR reaction (2% to 40%) as needed for your application. Adjusting the proportion of blood in the assay requires some modification to the standard amplicon recovery procedure, as outlined in the optimization parameters and protocol steps below.
- For analysis of non-human blood samples, first determine the amount of blood that can be included in the PCR reactions by including different amounts of the blood samples in PCR reactions that amplify well-characterized targets from plasmid DNA.

Optimization parameters

Amplification of challenging PCR targets, including GC-rich and long (>1kb) targets, may require optimization of PCR conditions including proportion of blood in the assay, use of DMSO, and adjustment of thermal cycling parameters.

Optimization for long (>1 kb) targets

- Begin optimization of long target PCR by including blood samples present at 2% to 5% (v/v) of the complete PCR reaction. For the 25-µL PCR reaction volume in the following protocol, start assay optimization using 0.5 µL to 1.25 µL of blood, adding PCR-grade water to bring the volume of each sample to 5 µL. Larger volumes of blood may be included in the assay if needed for a particular target, but additional optimization steps, such as DMSO inclusion, may be required.
- Amplification of some long targets may be improved by including DMSO in the PCR reaction mixture. If amplification appears inefficient, titrate DMSO in 1% increments in the range of 1% to 6% in the final PCR reaction, using the DMSO provided in the kit. Adjust the amount of water added to the reaction mixtures accordingly. DMSO may increase PCR error rates so should be avoided in cases where there is no benefit to yield or specificity.
- Use the specialized cycling condtions provided in Table 8 on page 18 for long PCR targets.

Optimization for GC-rich targets

- Begin optimization of GC-rich target PCR by including blood samples present at 5% to 20% (v/v) of the complete PCR reaction. For the 25-µL PCR reaction volume in the following protocol, start assay optimization using 1.25 µL to 5 µL of blood, adding PCR-grade water to bring the volume of each sample to 5 µL when required. Larger volumes of blood may be tested in the assay if indicated for a particular target, but higher proportions of blood in the assay may decrease performance of the system, making detection of challenging targets more difficult.

- Amplification of GC-rich targets may be improved by including DMSO in the PCR reaction mixture. If amplification appears inefficient, titrate DMSO in 1% increments in the range of 1% to 8% in the final PCR reaction, using the DMSO provided in the kit. Adjust the amount of water added to the reaction mixtures accordingly. DMSO may increase PCR error rates so should be avoided in cases where there is no benefit to yield or specificity.
- Use the specialized cycling condtions provided in Table 9 on page 18 for long PCR targets.

Step 1. PCR-amplify the target DNA from liquid blood samples

You can design SureDirect PCR assays for liquid blood samples using a variety of reaction volumes and blood sample volumes, as long as the provided 2× master mix makes up 50% of the final PCR reaction volume.

The example protocol below analyzes 5-µL blood samples in a 25-µL PCR reaction volume, for a final PCR reaction composition of 20% (v/v) blood. The volume of blood analyzed and the proportion of blood in the assay can be modified, if needed, by changing the reaction volume and amount of water added to the reaction mixture. Blood samples may be added to the PCR reaction at 10–40% of the final reaction volume with some modifications to post-PCR sample processing steps, as detailed in the protocol below.

Example 25-µl PCR assay using 5-µl blood samples

1. For runs that include the provided positive control, first dilute 1 µL of the 10× Control Primers + Template solution in 9 µL of PCR-grade water in a 1.5 mL Lo-Bind tube. Keep on ice.
2. Place 5 µL of each blood sample to be PCR-analyzed in wells of 8-well strip tubes.

   As appropriate, include a no-template control by placing 5 µL of PCR-grade water in an additional strip tube well.

   Positive control reactions should be set up by placing either 5 µL of PCR-grade water (for verifying performance of kit components) or 5 µL of blood sample (to analyze effects of blood sample factors on PCR performance) in an additional strip tube well.
3. Prepare a mixture of PCR master mix and PCR primers, as described in Table 5. Combine the reagents in a 1.5 mL Lo-Bind tube and keep on ice. Prepare the amount required for the number of samples and no-template controls in the run, plus excess.

   For positive control reactions, combine the reagents listed in Table 6 in a separate tube.

Table 5 Preparation of PCR master mix + primers for multiple test reactions

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 16 reactions (includes excess)
PCR-grade water	5.5 µL	46.75 µL	93.5 µL
SureDirect Blood PCR 2X Master Mix (MM)	12.5 µL	106.25 µL	212.5 µL
Forward Primer (10 µM)	1 µL	8.5 µL	17 µL
Reverse Primer (10 µM)	1 µL	8.5 µL	17 µL
Total	**20 µL**	**170 µL**	**340 µL**

NOTE

When amplifying GC-rich, long, or other difficult targets, results may be improved by adding DMSO to the PCR reaction mixture. See "Optimization parameters" on page 13 for suggested titration ranges. When adding DMSO to the mixture, decrease the amount of water added to the mixture accordingly.

Table 6 Preparation of positive control reaction mixture

Reagent	Volume for 1 reaction
PCR-grade water	6.5 µL
SureDirect Blood PCR 2X Master Mix (MM)	12.5 µL
1× Control Primers + Template dilution, prepared in step 1	1 µL
Total	**20 µL**

4 Mix by vortexing, then spin the tube briefly to collect the liquid.

5 Transfer 20 µL of the mixture prepared in Table 5 or Table 6 to the appropriate sample wells prepared in step 2.

6 Mix by vortexing and then spin the tube strips briefly to collect the liquid.

7 Place the tube strips in a thermal cycler and run the PCR program appropriate program for your target:

- For typical targets, including the positive control template and primer pair included with the kit, optimize PCR starting with the program in Table 7
- For long targets (>1 kb), optimize PCR starting with the program in Table 8
- For GC-rich targets, optimize PCR starting with the program in Table 9

For all target types, do the PCR using a heated lid. If your thermal cycler has multiple ramp rate options, use the standard ramp rate; **do not use a fast ramp rate setting**.

Table 7 Recommended PCR program for typical targets

Segment	Number of Cycles	Temperature	Time
1	1	90°C	5 minutes
2	30	95°C	30 seconds
		Primer T_m–5°C*	30 seconds
		72°C	1 minute†
3	1	72°C	5 minutes
4	1	4°C	Hold

* Use the annealing temperature appropriate for your primer pair. For the positive control provided with the kit, use 60°C.

† Yield of some amplicons close to 1 kb length may be improved by using a 75-second extension time.

Table 8 Recommended PCR program for targets >1 kb

Segment	Number of Cycles	Temperature	Time
1	1	90°C	5 minutes
2	35	94°C	45 seconds
		Primer T_m–5°C*	45 seconds
		72°C	1 minute per kb
3	1	72°C	5 minutes
4	1	4°C	Hold

* Use the annealing temperature appropriate for your primer pair.

Table 9 Recommended PCR program for GC-rich targets

Segment	Number of Cycles	Temperature	Time
1	1	90°C	5 minutes
2	30	98°C	30 seconds
		Primer T_m–5°C*	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

* Use the annealing temperature appropriate for your primer pair.

Step 2. Recover the amplicons from lysed cell debris

Exposing the blood samples to PCR conditions produces a viscous cell lysate material. In this step, the aqueous component of the lysate, including the amplicons, is recovered from the cell debris in the suspension by centrifugation.

Agilent recommends performing the centifugation steps below using a microcentrifuge rated at 15,000 rpm and equipped with a strip tube-compatible rotor, such as the Eppendorf 5424 benchtop microcentrifuge with strip-tube rotor accessory. Using this optimal equipment facilitates efficient recovery of the amplicon-containing supernatant from the substanial amount of cell debris released during this procedure. Alternatively, the procedure can be adapted to use a clinical centrifuge with a swinging-bucket rotor, using the modified procedure described on page 20.

1 Load the strip tubes in a microcentrifuge equipped with a strip tube rotor.

2 Centrifuge the strip tubes at 15,000 rpm for the time period appropriate for your sample composition, as shown in Table 10.

Table 10 Recommended centrifugation time

Percent Blood (v/v) in PCR Reaction	Centrifugation Time
up to 10%	5 minutes
10–20%	5–10 minutes
20%	10 minutes
20–40%	10–15 minutes
40%	at least 15 minutes

3 Immediately after the centrifugation period is complete, collect the supernatants using a pipette, transferring the liquid from each strip tube well to separate, fresh LoBind tubes.

CAUTION

It is important to collect the supernatant from the cell debris immediately after centrifugation. The supernatant may be reabsorbed by the viscous pellet when not immediately removed. If required, samples may be subjected to a second round of centrifugation.

Modified amplicon collection protocol using a clinical centrifuge

A clinical centrifuge with a swinging-bucket rotor, such as the Eppendorf 5804 centrifuge, may be used for the adapted amplicon recovery procedure, as described below.

1. Equip the centrifuge buckets with a 96-well plate adapter or similar.
2. Load the strip tubes in the 96-well plate adapter racks.
3. Centrifuge the strip tubes at 11,000 rpm for 45 minutes.
4. Immediately after the centrifugation period is complete, collect the supernatants using a pipette, transferring the liquid from each strip tube well to separate, fresh LoBind tubes.

CAUTION It is important to collect the supernatant from the cell debris immediately after the centrifugation step. The supernatant may be reabsorbed into the debris when not immediately removed. If required, samples may be subjected to a second round of centrifugation.

Step 3. Analyze the amplicons by gel electrophoresis

Analyze the presence of the target amplicon in the recovered supernatant using gel electrophoresis. Typical qualitative analysis conditions are electrophoresis of 10 µL of the supernatant using 1–3% agarose, or NuSieve agarose gels, depending on the PCR product size. Include a DNA ladder with fragments in the appropriate size range in one lane of each gel to verify the size of the amplicon(s) present in each sample.

Expected Results for the Positive Control

Amplification of the positive control target using the provided Control Pimers + Template produces a single 248-bp amplicon.

Analysis of 10 µL of the positive control reaction is expected to produce a readily-visualized band using standard laboratory gel processing protocols.

Use the same 10-µL volume for analysis of postive controls run in plain water samples and for analysis of supernatants recovered from centrifugation of postive controls run in blood samples.

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Analysis of Dried Blood Samples

For PCR analysis of dried blood samples using the SureDirect Blood PCR Kit, follow the instructions in this chapter.

For analysis of liquid blood samples, instead see Chapter 2 on page 11.

PCR primer considerations

Design PCR primers for analysis of blood samples using the following considerations:

- The SureDirect Blood PCR Kit can be used to amplify a variety of target sizes and composition profiles with some optimization of conditions. For assays that require the least optimization, however, design targets in the 200 to 400 bp range and with typical GC-content. The positive control reagents provided with the kit produce a 248-bp amplicon.
- Primers should have a calculated T_m value of approximately 63°C to 73°C. You can use the IDT OligoAnalyzer 3.1 tool, provided by the recommended primer vendor, to estimate primer T_m. The recommended annealing temperature for amplification using the SureDirect Blood PCR Kit is 5°C lower than the average primer T_m.

Before using the chosen primer pair for target amplification from blood samples, validate the specificity and yield of the PCR reaction using purified genomic DNA as template.

Preparation and storage of dried blood samples

- Blood samples to be analyzed with the SureDirect Blood PCR Kit in dried form should be prepared by applying liquid blood samples to paper supports. Agilent recommends using Whatman 903 Protein Saver Cards for this purpose.
- Blood samples collected with or without anticoagulant may be analyzed using the dried blood sample protocol.
- Using a pipette or capillary tube, apply 50 µL of each blood sample to the center of a circular spot marked on the sample card. Avoid touching the card with the pipette or capillary tube.
- After application, thoroughly dry the blood samples on the paper supports. Store the dried cards sealed in plastic bags with dessicant packets at room temperature or at –20°C.

- Just before analysis, punch out a 2-mm diameter disc containing the blood sample. Sterilize the punch tool between samples.

CAUTION To reduce the risk of sample loss, do not punch the discs from the sample cards in a hood with the fan on.

Optimization parameters

Amplification of challenging PCR targets, including GC-rich and long (>1kb) targets, may require optimization of PCR conditions including use of DMSO and adjustment of thermal cycling parameters.

Optimization for long (>1 kb) targets

- Amplification of some long targets may be improved by including DMSO in the PCR reaction mixture. If amplification appears inefficient, titrate DMSO in 1% increments in the range of 1% to 6% in the final PCR reaction, using the DMSO provided in the kit. Adjust the amount of water added to the reaction mixtures accordingly. DMSO may increase PCR error rates so should be avoided in cases where there is no benefit to yield or specificity.
- Use the specialized cycling condtions provided in Table 14 on page 29 for long PCR targets.

Optimization for GC-rich targets

- Amplification of GC-rich targets may be improved by including DMSO in the PCR reaction mixture. If amplification appears inefficient, titrate DMSO in 1% increments in the range of 1% to 8% in the final PCR reaction, using the DMSO provided in the kit. Adjust the amount of water added to the reaction mixtures accordingly. DMSO may increase PCR error rates so should be avoided in cases where there is no benefit to yield or specificity.
- Use the specialized cycling condtions provided in Table 15 on page 29 for long PCR targets.

Step 1. PCR-amplify the target DNA from dried blood samples

The example protocol below analyzes dried blood samples on 2-mm diameter filter discs in a 25-µL PCR reaction volume. You can modify the design of the SureDirect PCR assay, including changing the reaction volume, as long as the provided 2× master mix makes up 50% of the final PCR reaction volume.

1. For runs that include the provided positive control, first dilute 1 µL of the 10× Control Primers + Template solution in 9 µL of PCR-grade water in a 1.5 mL Lo-Bind tube. Keep on ice.
2. Add 5 µL of PCR-grade water into wells of 8-well strip tubes, filling enough wells for the number of dried blood samples in the run.

   As appropriate, add 5 µL of PCR-grade water to additional, empty wells for no-template control and positive control reactions.
3. Place 2-mm diameter filter discs containing the dried blood samples into the strip tube wells (one disc per well).
4. Prepare a mixture of PCR master mix and PCR primers, as described in Table 11. Combine the reagents in a 1.5 mL Lo-Bind tube and keep on ice. Prepare the amount required for the number of samples and no-template controls in the run, plus excess.

   For positive control reactions, combine the reagents listed in Table 12 in a separate tube.

Table 11 Preparation of PCR master mix + primers for multiple test reactions

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 16 reactions (includes excess)
PCR-grade water	5.5 µL	46.75 µL	93.5 µL
SureDirect Blood PCR 2X Master Mix (MM)	12.5 µL	106.25 µL	212.5 µL
Forward Primer (10 µM)	1 µL	8.5 µL	17 µL
Reverse Primer (10 µM)	1 µL	8.5 µL	17 µL
Total	**20 µL**	**170 µL**	**340 µL**

NOTE

When amplifying GC-rich, long, or other difficult targets, results may be improved by adding DMSO to the PCR reaction mixture. See "Optimization parameters" on page 25 for suggested titration ranges. When adding DMSO to the mixture, decrease the amount of water added to the mixture accordingly.

Table 12 Preparation of positive control reaction mixture

Reagent	Volume for 1 reaction
PCR-grade water	6.5 µL
SureDirect Blood PCR 2X Master Mix (MM)	12.5 µL
1× Control Primers + Template dilution, prepared in step 1	1 µL
Total	**20 µL**

5 Mix by vortexing, then spin the tube briefly to collect the liquid.

6 Transfer 20 µL of the mixture prepared in Table 11 or Table 12 to the sample wells containing blood samples in 5 µL of PCR-grade water (from step 3, above).

7 Mix well by vortexing and then spin the tube strips briefly to collect the liquid.

8 Place the tube strips in a thermal cycler and run the PCR program appropriate program for your target:

- For typical targets, including the positive control template and primer pair included with the kit, optimize PCR starting with the program in Table 13
- For long targets (>1 kb), optimize PCR starting with the program in Table 14
- For GC-rich targets, optimize PCR starting with the program in Table 15

For all target types, do the PCR using a heated lid. If your thermal cycler has multiple ramp rate options, use the standard ramp rate; **do not use a fast ramp rate setting**.

Table 13 Recommended PCR program for typical targets

Segment	Number of Cycles	Temperature	Time
1	1	90°C	5 minutes
2	30	95°C	30 seconds
		Primer T_m–5°C*	30 seconds
		72°C	1 minute†
3	1	72°C	5 minutes
4	1	4°C	Hold

* Use the annealing temperature appropriate for your primer pair. For the positive control provided with the kit, use 60°C.

† Yield of some amplicons close to 1 kb length may be improved by using a 75-second extension time.

Table 14 Recommended PCR program for targets >1 kb

Segment	Number of Cycles	Temperature	Time
1	1	90°C	5 minutes
2	35	94°C	45 seconds
		Primer T_m–5°C*	45 seconds
		72°C	1 minute per kb
3	1	72°C	5 minutes
4	1	4°C	Hold

* Use the annealing temperature appropriate for your primer pair.

Table 15 Recommended PCR program for GC-rich targets

Segment	Number of Cycles	Temperature	Time
1	1	90°C	5 minutes
2	30	98°C	30 seconds
		Primer T_m–5°C*	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

* Use the annealing temperature appropriate for your primer pair.

Step 2. Analyze the amplicons by gel electrophoresis

Analyze the presence of the target amplicon using gel electrophoresis. Typical qualitative analysis conditions are electrophoresis of 10 µL of each PCR-amplified sample using 1–3% agarose, or NuSieve agarose gels, depending on the PCR product size. Include a DNA ladder with fragments in the appropriate size range in one lane of each gel to verify the size of the amplicon(s) present in each sample.

Expected Results for the Positive Control

Amplification of the positive control target using the provided Control Pimers + Template produces a single 248-bp amplicon.

Analysis of 10 µL of the positive control reaction is expected to produce a readily-visualized band using standard laboratory gel processing protocols.

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In This Book

This guide contains information to run the SureDirect Blood PCR Kit protocol.

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