DxDirect™: Multiplexed Gene Expression Analysis Direct from DxCollect RNA Stabilized Blood

Compatible with DxTerity DxCollect™ Blood RNA Collection Tubes

User Manual

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Use Limitations

The DxDirect reagents are intended for Research Use Only.

DxDirect Reagent System Contents and Storage Conditions

The components of the DxDirect Reagent System and their recommended storage conditions are listed below. The DxDirect Reagent System is designed for use with magnetic plate washers and is available in 2 sizes—200 reactions and 1,000 reactions. Kit components have a shelf life of 6 months from date of receipt.

Store reagents according to labels on boxes.

Do not pool reagents or mix and match kit components from different lots.

Reagent Box 1: Store between -30°C and -15°C

Description	Size	Storage
DirectPrime—Universal amplification primers for up to 40 targets multiplexed assays	1 mL	-30°C to -15°C
DirectTaq—PCR Reaction Mix containing enzyme for 200 reactions	1 mL	-30°C to -15°C

Reagent Box 2: Store between 2°C and 8°C

Description	Size	Storage
DirectMix C—Proteinase K for 200 reactions	1 mL	2°C to 8°C
DirectBeads—Magnetic capture beads for 200 reactions	2 mL	2°C to 8°C
DirectReact—Reaction buffer for 200 reactions	2 mL	2°C to 8°C

DirectWash: Store between 2° to **25**°**C**

Description	Size	Storage
DirectWash—Wash buffer for 200 reactions	2 x 125 mL	2°C to 25°C

Equipment and Reagents Required

Required Material and Equipment	Recommended
DirectMix A	User supplied
DirectMix B	User supplied
PCR Thermal Cyclers with Hot Lids	PCR Thermal Cycler with a temperature range from 4.0°C to 99.9°C; maximum block ramp rate of 3.9°C⁄sec
Vortex Mixer	Any model
Centrifuge for 96-well Plates	Any model
Adjustable Volume Pipettes (single and 8-channel)	Any model
Handheld Magnetic Capture Plate	Life Technologies DynaMag™—96-well Side-Skirted Magnetic Plate (CAT # 120-27)
Lab Consumables	96-well 0.2 mL PCR plates; 8-well strip covers or plate tape seals; and reagent reservoirs

Recommended Capillary Electrophoresis Platforms

Model	Array	Polymer Formulation
Applied Biosystems (ABI) 3130	8- & 16-capillary array	POP-6 & POP-7
ABI 3500 and 3500XL	8- & 24-capillary array	POP-6 & POP-7
ABI 3730	48-capillary array	POP-6 & POP-7
ABI 3730XL	96-capillary array	POP-6 & POP-7

Technical Support

For questions regarding the DxDirect Assay, contact:

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Introduction

The DxDirect assay technology combines Chemical Ligation Probe Amplification (CLPA) technology with a proprietary blood stabilization buffer, DxCollect, to enable direct from sample analysis of genomic signatures. CLPA uses a robust, non-enzymatic chemical reagent system to enable the direct quantitative analysis of RNA and DNA in denaturing chemical environments like those experienced in stabilized, unpurified blood. Sample processing and multiplexed, end-point PCR is performed in a single reaction tube, and the final assay amplicon detection is accomplished using capillary electrophoresis instruments.

How DxDirect Works

DxDirect must be used in conjunction with probes designed by the user according to the DxDirect Probe Design User Manual and probe mixtures optimized according to the Assay Optimization Application Note

For every gene in the assay, a unique probe set is designed consisting of two DNA oligonucleotides, known as the Thiol Group Probe (S-Probe) and the Leaving Group Probe (L-Probe), that bind adjacently to one another and capture probes upstream or downstream from the target site (refer to Figure 1). Each S- and L-Probe pair is designed to contain a target-specific hybridization sequence and a unique identification sequence that enables size separation by CE. All S- and L-Probes contain upstream and downstream universal primer sequences to allow for multiplex PCR amplification of all ligation products.

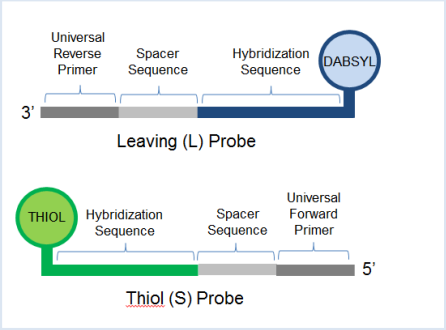


Figure 1. Design of S- and L-Probes

In the DxDirect method (refer to Figure 2), S- and L-Probes hybridize adjacently to one another leading to a chemical **LIGATION** reaction triggered by proximity of the Leaving (L) group to the Thiol (S) group. The ligation products are generated directly in the denatured sample; **CAPTURED** on magnetic beads to remove un-reacted probes; and then **AMPLIFIED** by PCR directly from the beads. The final amplified product is **DETECTED** by CE, based on final amplicon size. The assay is quantitative because the amount of each ligation product made during the DxDirect reaction is proportional to the concentration of its RNA target sequence, and the relative ratios of ligation products are maintained during PCR amplification, since amplification uses only a single PCR primer. Quantification is relative to one or more housekeeping genes in the assay.

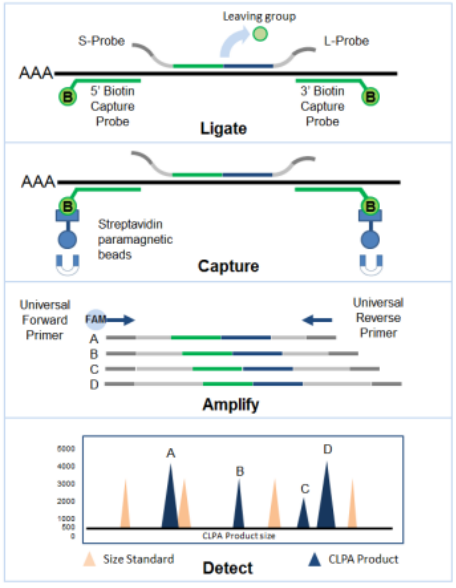


Figure 2. Basic workflow of the DxDirect method.

DxDirect Assay Quick Guide

Ligation

+ 15 µL DirectReact.
+ 50 µL of the DxCollect Blood.
+ 15 µL of the DirectMix A.
+ 20 µL of DirectMix B/DirectMix C.

Run **Ligation Program** on thermal cycler.

+ 5 µL pre-mixed DirectBeads.

Run **Incubation Program** on thermal cycler.

Capture

Place on magnetic capture plate for 2 minutes, then aspirate liquid.

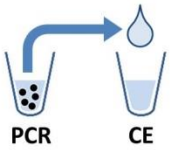
+ 180 µL DirectWash and resuspend beads. Place on magnetic capture plate for 2 minutes, then aspirate DirectWash solution.

Amplify

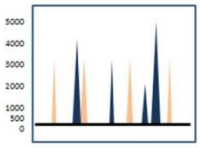
+ 10 µL of PCR Mastermix.

Run **Amplification Program** on thermal cycler.

Detect



+ 18 µL of CE Master Mix into new CE Plate.
+ 2 µL of PCR product.



Detect PCR products by CE.

☐ 1. Add 15 µL DirectReact to each well of the reaction plate.
☐ 2. Add 50 µL of the DxCollect Blood Sample to each well of the reaction plate—MIX WELL.
☐ 3. Add 15 µL of the DirectMix A to each well of the reaction plate.
☐ 4. Add 20 µL of pre-mixed DirectMix B/DirectMix C to each well of the reaction plate—MIX WELL.
☐ 5. Incubate the reaction plate in a PCR Thermal Cycler:

Thermal Cycler Protocol—Ligation		
Step	**Temperature**	**Duration**
1	55°C	5 min
2	80°C	10 min
3	55°C	45 min

☐ 6. Remove the reaction plate and add 5 µL of pre-mixed M270 Beads to each well of the reaction plate—MIX WELL.
☐ 7. Incubate the reaction plate in a PCR Thermal Cycler:

Thermal Cycler Protocol—Product Capture		
Step	**Temperature**	**Duration**
1	55°C	15 min

☐ 8. Place the plate on the magnetic plate for at least 30 seconds.
☐ 9. Remove all supernatant and remove the plate from magnetic plate.
☐ 10. Add 180 µL DirectWash to each well and re-suspend the beads by pipetting.

Repeat 3 times

☐ 11. Prepare the PCR Master Mix (1:1 mix of DirectPrime and DirectTaq).
☐ 12. Remove all supernatant from the plate and add 10 µL of PCR Mastermix to each well of the reaction plate—cap wells and vortex for 5 to 10 seconds.
☐ 13. Centrifuge the reaction plate and perform PCR:

Thermal Cycler Protocol—PCR			
Step		**Temperature**	**Duration**
Hot Start		95°C	2 min
3-Step Cycling	Denaturation	95°C	10 sec
	Annealing	57°C	20 sec
	Extension	72°C	20 sec
# Cycles	30	N/A	
Hold		4°C	∞

☐ 14. Run the final products on CE according to the manufacturer's instructions.

Detailed Procedure

1. Sample Preparation

DxDirect has been optimized to work with DxCollect RNA Blood Collection Tubes.

Blood samples collected in DxCollect RNA Blood Collection Tubes are stable at room temperature for up to 7 days prior to processing. If blood samples are to be stored longer than 7 days, then the DxCollect Tubes should be stored in a -20°C freezer until processing. Prior to using frozen samples, place the tubes on the bench top for at least 60 minutes to bring samples to room temperature. Once at room temperature, vortex the tubes gently and proceed with the procedure.

2. Hybridization and Ligation

Prior to starting the procedure, prepare both DirectMix A and DirectMix B; please refer to Appendix A and follow the directions.

1. Dispense the appropriate amount of DirectReact reagent into a labeled 1.5 mL microcentrifuge tube* for the total number of tests to be performed (15 µL per reaction).
2. Dispense the appropriate amount of DirectMix A into a labeled 1.5 mL microcentrifuge tube* for the total number of tests to be performed (15 µL per reaction).
3. Mix DirectMix B/DirectMix C by combining 15 µL of DirectMix B and 5 µL DirectMix C per reaction of both reagents into a labeled 1.5 mL microcentrifuge tube*.

* Alternatively, the reagents can be dispensed into 8-well strip microfuge tubes to facilitate pipetting with a mutli-channel pipette into the 96-well reaction plate.

4. In a 96-well PCR plate, dispense 15 µL of DirectReact per well into the appropriate number of wells. (Keep in mind the total number of wells will depend on the number of replicates per sample). Per well:
    a. Add 50 µL of DxCollect stabilized blood sample into the appropriate wells and mix by pipetting 2–3 times.
    b. Add 15 µL DirectMix A and 20 µL DirectMix B/C mixture to all reaction wells.
    c. Mix the reactions in each well by pipetting up and down 5 times with a multi-channel pipette set at 20 µL.
5. Seal the 96-well reaction plate and place the plate in a PCR thermal cycler pre-programmed to perform one cycle each of the following 3 incubation steps:

Thermal Cycler Protocol—Ligation		
Step	Temperature	Duration
1	55°C	5 min
2	80°C	10 min
3	55°C	2 hr 45 min

3. Target Capture

1. At the end of the 55°C incubation, remove the plate from the thermocycler and after carefully opening the sealed plate, add 5 µL of the DynaBead Magnetic Beads into each reaction using a multi-channel pipette. Mix the reactions in each well by pipetting up and down 5 times with a multi-channel pipette set at 20 µL. Return the reaction plate to the thermal cycler and incubate for an additional 15 minutes at 55°C.
2. At the end of 15 minutes, remove the reaction plate and place onto a magnetic bead capture plate for at least 30 seconds. During capture, fill a reagent reservoir suitable for multi-channel pipetting with DirectWash solution.
3. Remove all ligation reaction solution from the sample plate using an 8-channel 200 µL pipette set to 180 µL and discard the solution into a biohazard waste container. Avoid pipetting out the beads.

NOTE: It is important that all of the reaction mixture is removed from the reaction plate. Carry-over of the reaction mixture may result in subsequent assay failures. Visually inspect the bottom of the plate to ensure that all liquid is removed from all reaction wells. If any liquid is visible in a well, place the plate back on the magnetic plate and pipette out the remaining liquid.

4. After removing the reaction solution, visually verify that the beads are on the side of each reaction well. If beads are not observed in the wells, this indicates loss of the beads/ligation reaction product, which will result in low signals.
5. Take the reaction plate off the magnetic plate, place the plate on a 96-well plate holder, and add 180 µL of DirectWash into all reaction wells using a multi-channel pipette. Gently re-suspend the beads by pipetting the solution up and down two times and return the plate to the magnetic capture plate.

NOTE: Avoid introducing bubbles by gently pipetting the bead mixture during resuspension.

6. Repeat for a total of 3 washes. At the end of the third wash, do NOT remove the wash solution from the beads.

NOTE: Be sure to visually confirm the magnetic capture of beads before discarding the supernatant between washes.

4. Amplification

1. Prepare the DirectTaq PCR Master Mix by combining 5 µL of DirectTaq and 5 µL of DirectPrime for a total volume of 10 µL per reaction.

2. Return the reaction plate to the magnetic capture plate for at least 30 seconds and remove the final wash solution from the reaction plate. Visually inspect the bottom of the plate to ensure that all liquid is removed from all wells. If any liquid is visible in a well, place the plate back on the magnetic plate and aspirate as much remaining wash liquid as possible.
3. Remove the reaction plate from the magnetic capture plate and immediately add 10 µL of the prepared DirectTaq PCR Master Mix into each well.

NOTE: Dispensing the DirectTaq PCR Master Mix directly onto the magnetic beads makes it easier to re-suspend the beads with minimal effort.

4. Cap or seal all reaction wells in preparation for PCR. Re-suspend the magnetic beads by vortexing the plate at a medium-high setting for 5 s to 15 seconds and briefly centrifuge to pellet the beads. Visually inspect the bottom of the plate to ensure that all beads are immersed in the PCR Master Mix. If any beads adhere to the sides of the wells exposed to air, vortex the plate at medium-high setting for 5 seconds and centrifuge the plate at medium speed for 5 seconds.
5. Transfer the plate to a PCR thermal cycler with a hot lid pre-programmed to the following thermal cycling conditions.

Thermal Cycler Protocol—PCR			
Step		Temperature	Duration
Hot Start		95°C	2 min
3-Step Cycling; 30 cycles	Denaturation	95°C	10 sec
	Annealing	57°C	20 sec
	Extension	72°C	20 sec
	Stop	4°C	Hold

6. After thermal cycling is completed, remove the reaction plate and briefly centrifuge. Transfer the reaction plate to the magnetic capture plate to capture the beads for at least 30 seconds.

5. Detection

1. Prepare sufficient quantities of CE master mix by combining 0.5 µL of the Size Standard and 17.5 µL of Formamide solutions per reaction.
2. Dispense 18 µL of CE Master Mix solution into the appropriate number of wells for the CE analysis plate.
3. Transfer 2 µL of final PCR reaction product from each reaction well of the reaction plate to the CE analysis plate while it is still on the magnetic plate. Seal the plate with a plate septum. Vortex the CE analysis plate at medium speed for 5 seconds and briefly centrifuge.

NOTE: Avoid transferring magnetic beads during pipetting. They may interfere with the injection process during CE.

4. Visually inspect the bottom of the CE plate to ensure that no bubbles are present at the bottom of any sample well. If any bubbles are observed, re-centrifuge the plate.
5. Run samples by CE according to manufacturer's user manual. For the ABI-3500 Genetic Analyzer, the following settings are recommended:

Description	Recommendation
Assay	Standard Fragment Analysis
Polymer type	POP-6 or POP-7
Capillary Array	50 cm
Oven temperature	60°C (default)
Sample Injection Time	30 sec
Sizing standard	GeneScan™ 600 LIZ® Size Standard v2.0

DxDirect Assay Worksheet: Ligation and Target Capture

Pre-Dispense of DirectReact in Preparation for Addition to Samples Number of reactions N = _____			
Item Description	**Volume per Reaction, (µL)**	**Volume Required, (µL)**	**Total Volume to Add (µL)**
DirectReact	15	15 X 1.2 X N	
Dispense into a 1.5 mL microtube or 8-microtube strip		Total Volume ÷ 8.35	

Pre-Dispense of DirectMix A in Preparation for Addition to Samples Number of reactions N = _____			
Item Description	**Volume per Reaction, (µL)**	**Volume Required, (µL)**	**Total Volume to Add (µL)**
DirectMix A	15	15 X 1.2 X N	
Dispense into a 1.5 mL microtube or 8-microtube strip		Total Volume ÷ 8.35	

Pre-Dispense of DirectMix B/C in Preparation for Addition to Samples Number of reactions N = _____			
Item Description	**Volume per Reaction, (µL)**	**Volume Required, (µL)**	**Calculated Volume to Add (µL)**
DirectMix B	15	15 X 1.2 X N	
DirectMix C	5	5 X 1.2 X N	
Total Volume	20	20 X 1.2 X N	
Dispense into a 1.5 mL microtube or 8-microtube strip		Total Volume ÷ 8.35	

Pre-Dispense of DirectBeads in Preparation for Addition to Samples Number of reactions N = _____			
Item Description	**Volume per Reaction, (µL)**	**Volume Required, (µL)**	**Total Volume to Add (µL)**
DirectBeads	5	5 X 1.2 X N	
Dispense into a 1.5 mL microtube or 8-microtube strip		Total Volume ÷ 8.35	

DxDirect Assay Worksheet: Amplification Setup

Pre-Dispense of PCR Master Mix in Preparation for Addition to Beads			
Item Description	**Volume per Reaction, (µL)**	**Volume Required, (µL)**	**Calculated Volume to Add (µL)**
DirectTaq	5	5 X N X 1.2 =	
DirectPrime	5	5 X N X 1.2 =	
Total Volume	10	10 X N X 1.2 =	
Dispense into a 1.5 mL microtube or 8-microtube strip		Total Volume ÷ 8.35	

Preparation of Formamide/Size Standard mixture			
Item Description	**Volume per Reaction, (µL)**	**Volume Required, (µL)**	**Calculated Volume to Add (µL)**
Formamide	17.5	17.5 X N X 1.2 =	
GeneScan 600 LIZ Size Standard v2.0	0.5	0.5 X N X 1.2 =	
Total Volume	18	18 X N X 1.2 =	
Dispense into a 1.5 mL microtube or 8-microtube strip		Total Volume ÷ 8.35	

Reagent Volumes for DxDirect Reactions (includes 20% extra volume)						
Formulation	**Reagents**	**Volume for 1 reaction**	**Volume for 24 reactions**	**Volume for 48 reactions**	**Volume for 72 reactions**	**Volume for 96 reactions**
NA	DirectReact	15 µL	432 µL	864 µL	1296 µL	1728 µL
NA	DirectMix A	15 µL	432 µL	864 µL	1296 µL	1728 µL
DirectMix B/C	DirectMix B	15 µL	432 µL	864 µL	1296 µL	1728 µL
	DirectMix C	5 µL	144 µL	288 µL	432 µL	576 µL
NA	M270 Beads	5 µL	144 µL	288 µL	432 µL	576 µL
PCR Master Mix	DirectTaq	5 µL	144 µL	288 µL	432 µL	576 µL
	DirectPrime	5 µL	144 µL	288 µL	432 µL	576 µL
CE Loading Master Mix	Formamide	17.5 µL	504 µL	1008 µL	1512 µL	2016 µL
	LIZ Size Standard	0.5 µL	14.4 µL	28.8 µL	43.2 µL	57.6 µL

Appendix A

General Guidelines for Preparation of DirectMix A and B

Before performing the DxDirect assay, DirectMix A and B must be prepared beforehand.

DirectMix A and B contain the probes that are used in the assay and are custom designed for your specific assay. For designing DxDirect probes (L-, S-, and Target Capture (TC)-Probe sets), please refer to the separate manual on probe design and the use of the AlleleID™ software (Premiere Biosoft).

DirectMix A contains S-probes in a diluent of 1XTE/1mM DTT.

DirectMix B contains L-probes plus TC-probes in a diluent of 1XTE buffer.

When analyzing DxDirect assays on the ABI CE platforms, it is preferable that gene-specific amplicons generate peak heights of at least 500 Relative Fluorescent Units (RFUs). Signals below 500 RFU can result in higher technical variability. Typically, the upper range of signaling for any given fragment should be a peak height of 10,000 to 15,000 RFU. The preferred upper limit of peak heights will depend on whether the gene is a normalizer or is expected to be up or down regulated. If signals are over 15,000 RFU, there is a risk that the peak height will be off-scale (over 28,000 RFU). As a threshold, any signals lower than 100 RFU should be excluded from the analysis as background.

For initial testing, the recommended final (in-assay) concentration is 200 pM for S- and L- probes and 400 pM for TC-probes. If gene-specific signals generated from the assay are too low, then the S-/L-probe concentration can be increased up to 800 pM. In the case of signals that are too high, the S-/L-probe concentrations can be lowered to as low as 50 pM. If a further decrease in signal is desired, an S-Probe Attenuator (SA-) Probe can be used (for further information see “DxDirect: Use of Attenuated S-Probes to Modulate Signal Magnitude for Specific Genes” Application Note available from DxTerity Technical Support). The concentrations of matched S- and L-probes should always be the same. In the case of attenuators, the concentration of an L-probe for any given gene should be equal to the sum of the concentration of the respective S-probe plus SA-probe.

In addition, it is also possible to modulate all signals in a given sample by changing the CE injection time. This results in a reduction of raw signals proportional to the change in injection time. For example, decreasing the CE injection time from 30 seconds to 15 seconds will result in a 50% decrease in the peak heights of all peaks (including the LIZ internal Size standard).

Quantification of DxDirect Probes

Upon receiving your probe sets, it is recommended that you confirm the concentration for each oligo by UV absorbance analysis. Measure the absorbance value of the probes at 260 nm and calculate the concentration of the stock probe concentration using the extinction coefficient value provided with the oligos.

Creation of Daughter Tubes

Dilute 100 µL of 10 µM stock probe with 900 µl of diluent to create 1 µM working stocks of all probes. For S-probes, use a diluent of 1X TE/1 mM DTT. For TC- and L-probes, use a diluent of 1X TE.

Formulation Directions for DirectMix A

NOTE: The probes in DirectMix A are at a concentration 6.67-fold higher than in the final mix. Therefore, if the desired final in-assay concentration of a probe is 100pM, the probe should be formulated in DirectMix A at a concentration of 667 pM.

1) Determine the volume of DirectMix A required. 15µL of DirectMix A is required for a single reaction. Therefore, 15.0 mL of DirectMix A is sufficient for approximately 1,000 reactions.
2) Calculate the volumes of the 1 µM daughter tube S-probes (and SA probes if required) that should be added to obtain the desired final concentrations. A good starting point is an in-reaction concentration of 200 pM S-probe per gene.
3) Add the calculated volume of each probe into an appropriately sized container.
4) After all probes have been added (and **before** the diluent is added), heat-activate the S-probe mix in a thermal cycler for 2 minutes at 95˚C.
5) Transfer the S-probes back to the container used for formulation and add the appropriate volume of diluent (1X TE/1 mM DTT).

Formulation Directions for DirectMix B

1) Determine the volume of DirectMix B required. 15 µL of DirectMix B is required for a single reaction. Therefore, 15.0 mL of DirectMix B is sufficient for approximately 1,000 reactions.
2) Calculate the volumes of the 1 µM daughter tube L-probes and TC-probes that should be added to obtain the desired final concentrations. A good starting point is an in-reaction concentration of 200 pM L-probe per gene and 400 pM TC-probe per gene).
3) Add the calculated volume of each probe into an appropriately sized container.
4) Add the appropriate volume of diluent (1X TE).

Contact Information

For technical questions regarding the DxDirect Assay and Probe Design, contact:

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For ordering or general questions regarding DxDirect or DxCollect products, contact:

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