ambion®
by life technologies™

Single Cell-to-CT™ Kit

Protocol

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TRADEMARKS

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Contents

About This Guide

Purpose

The *Single Cell-to-CT*™ Kit Protocol provides instructions and troubleshooting information for using the Single Cell-to-CT™ Kit.

Safety information

Note: For general safety information, see this section and Appendix B, "Safety" on page 21. When a hazard symbol and hazard type appear by an instrument hazard, see the "Safety" Appendix for the complete alert on the instrument.

Safety alert words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT, CAUTION, WARNING, DANGER**—implies a particular level of observation or action, as defined below:

IMPORTANT! – Indicates information that is necessary for proper instrument operation or accurate chemistry kit use.

CAUTION! – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING! – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER! – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

SDSs

The Safety Data Sheets (SDSs) for any chemicals supplied by Applied Biosystems or Ambion are available to you free 24 hours a day. For instructions on obtaining SDSs, see "SDSs" on page 22.

IMPORTANT! For the SDSs of chemicals not distributed by Applied Biosystems or Ambion contact the chemical manufacturer.

Single Cell-to-CT™ Kit

Product information

Purpose of the product

The Single Cell-to-CT™ Kit allows customers to analyze single or low cell numbers (up to 10 cells) through a validated qRT-PCR workflow.

Kit contents and storage

The Single Cell-to-CT™ Kit is available in two sizes: 50 reactions (PN 4458237) and 400 reactions (PN 4458236). Each reaction contains reagents sufficient for 1 sample prep, 1 reverse transcription reaction, 1 preamplification reaction, and 5 PCR reactions.

Component	Quantity		Storage Conditions
	50 rxns PN 4458237	400 rxns PN 4458236	
Single Cell Lysis Solution	0.5 mL	4.0 mL	4 °C
Single Cell DNase I	50 µL	400 µL	-20 °C
Single Cell Stop Solution	50 µL	400 µL	-20 °C
Single Cell VILO™ RT Mix	150 µL	1.2 mL	-20 °C
Single Cell SuperScript® RT	75 µL	600 µL	-20 °C
Single Cell PreAmp Mix	265 µL	2.1 mL	-20 °C
TaqMan® Gene Expression Master Mix	5.0 mL	50 mL	4 °C

Materials and equipment required

For the SDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

Item	Source
1X TE Buffer, pH 8.0	Applied Biosystems
Nuclease-free water	Applied Biosystems
Real-Time PCR instrument	Applied Biosystems
TaqMan® Gene Expression Assays	Applied Biosystems
Thermal cycler	Applied Biosystems
RealTime StatMiner® Software	Integromics
Microcentrifuge	Major laboratory suppliers (MLS)
Microcentrifuge tubes	Applied Biosystems
Pipette tips, nuclease-free	Applied Biosystems
Pipettors, positive displacement or air-displacement	MLS
Vortexer	MLS

Workflow

The Single Cell-to-CT™ Kit workflow is comprised of 4 functional steps: cell lysis, reverse transcription, cDNA pre-amplification and Real-Time PCR as outlined in the following schematic.

Add 1-10 cells to 10 µL of Single Cell Lysis/Dnase I solution

Incubate 5 min at room temp

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Add 1 µL of Stop Solution to lysis reaction (rxn volume 11 µL)

Incubate 2 min at room temp or store at -20 °C

(Stopping point)

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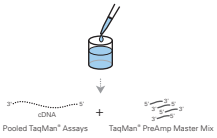
Add 4.5 µL of RT Mix

(rxn volume 15 µL)

Incubate for 10 min at 25 °C
Incubate 60 min at 42 °C
Incubate 5 min at 85 °C

(Stopping point)



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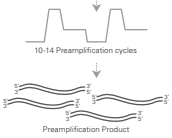
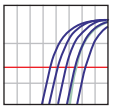
Add 11 µL of PreAmp Mix/pooled TaqMan assays
(rxn volume 26 µL)

Hold 95 °C for 10 minutes, then 14 cycles:
95 °C for 15 sec
60 °C for 4 min



(Stopping point)



↓

Add 25 µL of diluted preamplified product into a qPCR

Prepare the samples for Real-Time PCR

Perform single-cell lysis

1. Add 1 µL Single Cell DNase I to 9 µL Single Cell Lysis Solution.

Component	Volume for each reaction
Single Cell Lysis Solution	9 µL
Single Cell DNase I	1 µL
Total Single Cell Lysis mix for each reaction	**10 µL**

2. Add 1–10 cells to the 10 µL Single Cell DNase I/Single Cell Lysis Solution (no mixing required), then incubate at room temperature for 5 minutes. Samples can be incubated up to 30 minutes, or stored at -20 C (freeze/thaw up to 5 items) with no harmful effects.

3. Add 1 µL Single Cell Stop Solution (no mixing required), incubate at room temperature for 2 minutes, then place the sample on ice. Samples can be incubated up to 30 minutes, or stored at -20 C (freeze/thaw up to 5 items) with no harmful effects.

STOPPING POINT The total sample volume of cell lysate is 11 µL.

Perform reverse transcription

1. Prepare sufficient RT reaction mix for all samples, then add 4.5 µL to each lysed cell sample:

Component	Volume for each reaction
Single Cell VILO™ RT Mix	3.0 µL
Single Cell SuperScript® RT	1.5 µL
Total RT reaction mix	**4.5 µL**

2. Perform reverse transcription in a thermal cycler.

Temp	Time
25 °C	10 min
42 °C	60 min
85 °C	5 min

STOPPING POINT The total sample volume of preamplified cDNA is 15.5 µL.

Samples can be stored at -20 C (freeze/thaw up to 5 times) with no harmful effects.

Perform preamplification

1. Pool the TaqMan® Gene Expression Assays for your targets of interest, then dilute the pooled assays using 1× TE Buffer, pH 8.0 so that each assay is at a final concentration of 0.2×.

2. Prepare sufficient PreAmp reaction mix, then add 11 µL to each reverse-transcribed sample:

Component	Volume for each reaction
Single Cell PreAmp Mix	5 µL
0.2× pooled TaqMan® Gene Expression Assays	6 µL
Total PreAmp reaction mix	**11 µL**

3. Perform preamplification in a thermal cycler:

Stage	Step	Temp	Time
Holding	Enzyme activation	95 °C	10 min
Cycling (14 cycles)	Denature	95 °C	15 sec
	Anneal/extend	60 °C	4 min
Holding	Enzyme Deactivation	99 °C	10 min

4. Place the tubes on ice or store at –20 °C.

STOPPING POINT The total sample volume of preamplified cDNA is 26.5 µL.

Samples can be stored at -20 C (freeze/thaw up to 5 items) with no harmful effects.

Perform Real-Time PCR

Dilute the preamplified product and prepare the amplification reactions

1. Prepare a 1:20 dilution of preamplified products with 1× TE Buffer, pH 8.0 before performing Real-Time PCR. Refer to the dilution table below for guidance. The entire sample does not need to be diluted.

Component	Volumes for 1:20 dilution			
Preamplified product volume	5 µL	10 µL	25 µL	26.5 µL
1× TE Buffer, pH 8.p	95 µL	190 µL	475 µL	503.5 µL
Total Sample Volume	100 µL	200 µL	500 µL	530 µL

2. Prepare the Reaction Plate or array:
   - Using 96-well or 384-well reaction plates:

Component	Volume for each reaction	
	96-well plate	**384-well plate**
2× TaqMan® Gene Expression Master Mix	25.0 µL	10.0 µL
Preamplified product diluted 1:20 with 1× TE Buffer, pH 8.0	10.0 µL	4.0 µL
20× TaqMan® Gene Expression Assay	2.5 µL	1.0 µL
Nuclease-free water	12.5 µL	5.0 µL
Total volume for each reaction	**50.0 µL**	**20.0 µL**

   - Using a TaqMan® Array:

Component	Volume for a full array
2× TaqMan® Gene Expression Master Mix	450 µL
Preamplified product diluted 1:20 with 1× TE Buffer, pH 8.0	450 µL
Total volume for the array	**900 µL**

Note: The total sample volume will vary depending on the format chosen:
- 96-well is 50 µL
- 384-well is 20 µL
- TaqMan array is 900 µL

Amplify the cDNA using a Real-Time PCR system

The following table illustrates how the sample is run on the 7900 instruments.

Stage	Step	Temp	Time
Holding	UDG incubation	50 °C	2 min
Holding	Enzyme activation	95 °C	10 min
Cycling (40 cycles)	Denature	95 °C	5 sec
	Anneal/extend	60 °C	1 min

Analyze the results

- Use an automatic baseline and set the threshold to 0.2.
- Review the amplification plots and remove outliers.
- Omit samples that are undetectable for all assays tested.
- Use RealTime StatMiner® Software to perform differential expression analysis.

Troubleshooting

The following table includes details about common issues and their respective solutions.

Observation	Possible cause	Recommended action
No PCR Product or Unexpected PCR Products	There were problems with adding or mixing the Stop Solution.	Components in the Lysis Solution may inhibit RT-PCR if they are not fully inactivated by the Stop Solution. • Add the Stop Solution directly to the lysate, in other words, touch the lysate with the opening of the pipet tip when adding the Stop Solution to make sure that the entire 1µL of Stop Solution is added to each sample. • Mix by pipetting up and down five times.
	RNA was degraded before starting the procedure.	To avoid RNA degradation, keep cells in PBS on ice before starting the cell lysis procedure. Take cells off ice just prior to adding Lysis Solution.
	RNase in the sample was not completely activated.	Too many cells were used in the lysis reaction. If too many cells per sample are used in the procedure, the RNase in the sample may not be totally inactivated and/or cellular components or debris could inhibit reverse transcription or PCR. • Generally 1-10 cells can be used successfully in the Cells-to-CT™ procedure, but if RT or PCR fails, try using fewer cells. Too much PBS was left on the cells, diluting the Lysis Solution. If > 1 µL of PBS remains in samples when the Lysis Solution is added, the Lysis Solution may be too dilute to fully inactivate cellular RNases. To avoid this, remove as much PBS as possible before adding Lysis Solution to the cells.
	Lysates sat too long before going into RT	Do not allow lysates to sit longer than 30 min at room temperature once the Lysis Solution has been added or 20 min after Stop Solution has been added. Either freeze the lysates at –20 °C or –80 °C, or start the RT reactions. Alternatively, lysates can be safely stored on ice for up to 2 hr after lysis.

Observation	Possible cause	Recommended action
No PCR product *(Continued)*	Sample does not contain the target RNA	Negative results are often difficult to confirm as valid. Consider running the following experiments before concluding that the sample does not contain the RNA of interest: • Verify that the TaqMan® Gene Expression Cells-to-CT™ procedure is working by including XenoRNA™ Control (from the TaqMan Cells-to-CT Control Kit, sold separately) in the sample. Dilute the Xeno RNA 1:10 with NF-water then add 1 uL after adding Stop Solution. Then use the XenoRNA TaqMan Gene Expression Assay to amplify a XenoRNA target. If product is generated in the XenoRNA amplification, but no product is seen in the PCR for the gene of interest, then it is possible that the RNA of interest is not expressed in the cells and/or is undetectable with this procedure. • Try lysing 10 cells in 10 uL Lysis/ Dnase /Solution by using a live/dead stain. • Verify that a live cell is in the sample by using a a live/ dead stain. • There may not be a cell in the sample. This can be confirmed if multiple genes show no signal. • Check that the PCR for your target works with your PCR primers, reagents, and equipment by using cDNA generated from purified RNA from the same source (or a similar one) in PCR. If the amplification does not give good results using cDNA from purified RNA, it will not work with Cells-to-CT lysate.
	There were problems with the preamplification	With the exception of assays for 18S rRNA, be sure to use the same assays for the preamplification and the Real-Time PCR. Otherwise levels of gene expression cannot be compared. **Note**: It is important to exclude 18S assays from the preamplification pool, because the 18S rRNA is so highly expressed that its amplification would deplete the PCR reagents and other targets would not be amplified to any significant degree. • Make sure that preamplification is for only 14 cycles. • Preamplification reaction products must be diluted before using them in PCR

Ordering Information, Related Documentation, and Support

How to order

For information on the Single Cell-to-CT™ Kits, go to the Applied Biosystems website at **www.appliedbiosystems.com** and select:

Products ▸ Real-Time PCR ▸ Reagents, & Kits ▸ RT-PCR Directly from Cells ▸ Single Cell-to-CT™ Kit

Item	Source
Single Cell-to-CT™ Kit, 50 reactions	Applied Biosystems PN 4458237
Single Cell-to-CT™ Kit, 400 reactions	Applied Biosystems PN 4458236
Single Cell Lysis Kit	Applied Biosystems PN 4458235

Optional materials and equipment not included

Equipment

Item	Source
Thermal Cycler for reverse transcription: • Veriti® 96-Well Thermal Cycler • GeneAmp® PCR System 9700 • Compatible with any thermal cycler	Applied Biosystems
Real-time PCR instrument: • Applied Biosystems 7900HT Fast Real-Time PCR System • Compatible with any real-time instrument that reads fluorophores	Applied Biosystems
Countess® Automated Cell Counter	Invitrogen PN C10227
BD FACSAria Flow Cytometer	BD Biosciences
Pipettors, nuclease-free	MLS
Pipette tips, nuclease-free	MLS
Microcentrifuge tubes, nuclease-free	MLS

Item	Source
96-well plates, U-bottom	MLS
Flasks, tissue culture	MLS
Water bath	MLS

Reagents

For the SDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

Item	Source
Water, RT-PCR grade	Ambion PN 9935
PBS, 10×	Ambion PN 9624
Live/Dead® Viability / Cytotoxicity Kit for Mammalian Cells	Invitrogen PN L-3224
0.05% Trypsin-EDTA (1×)	GIBCO PN 633378
Cell culture media	MLS

Related documentation

The following related documents are available:

Document	Part number
Single Cell-to-CT™ Kit Protocol	4458356
Single Cell-to-CT™ Kit Quick Reference Card	4458357

Portable document format (PDF) versions of these documents are available on the Applied Biosystems web site at **www.appliedbiosystems.com**.

Obtaining support

For the latest services and support information for all locations, go to:

www.appliedbiosystems.com

At the Applied Biosystems web site, you can:

- Access worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.
- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents.
- Download PDF documents.
- Obtain information about customer training.
- Download software updates and patches.

Safety

This appendix covers:

Chemical safety

General chemical safety

Chemical hazard warning

WARNING! CHEMICAL HAZARD. Before handling any chemicals, refer to the Safety Data Sheet (SDS) provided by the manufacturer, and observe all relevant precautions.

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About SDSs" on page 22.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the SDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the SDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

SDSs

About SDSs

Chemical manufacturers supply current Safety Data Sheets (SDSs) with shipments of hazardous chemicals to new customers. They also provide SDSs with the first shipment of a hazardous chemical to a customer after an SDS has been updated. SDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new SDS packaged with a hazardous chemical, be sure to replace the appropriate SDS in your files.

Obtaining SDSs

The SDS for any chemical supplied by Applied Biosystems is available to you free 24 hours a day. To obtain SDSs:

1. Go to **www.appliedbiosystems.com**, click **Support**, then select **SDS**.
2. In the Keyword Search field, enter the chemical name, product name, SDS part number, or other information that appears in the SDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose

Note: For the SDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

Biological hazard safety

General biohazard

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **www.cdc.gov/biosafety/publications/index.htm**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **www.access.gpo.gov/ nara/cfr/waisidx_01/ 29cfr1910a_01.html**).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at:

www.cdc.gov

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Technical Resources and Support
For the latest technical resources and support information for all locations, please refer to our Web site at
www.appliedbiosystems.com/support