Cell input optimization for SYBR™ Green Fast Advanced Cells-to-C_T™ Kit

Catalog Numbers A35379, A35380, A35381

Publication Number MAN0017931 Revision B.0

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Experiment description

Purpose

The purpose of this experiment is to identify the maximum number of cells to use in SYBR™ Green Fast Advanced Cells-to-C_T™ reactions. Using too many cells can result in inefficient cell lysis and RT-PCR inhibition, and the maximum number of cells varies according to the cell type.

For the complete workflow and protocols refer to *SYBR™ Green Fast Advanced Cells-to-C_T™ Kit User Guide* (Pub. No. MAN0017755).

Experimental overview

In this experiment, cells are serially diluted and lysed following the normal protocol. If you have the SYBR™ Green Cells-to-C_T™ Control Kit, we recommend adding Xeno™ RNA Control to the Stop Solution used to prepare Cells-to-C_T™ lysates. The lysates are then subjected to real-time RT-PCR for an endogenous control gene, such as β-actin (a set of PCR primers for β-actin is included in the SYBR™ Green Cells-to-C_T™ Control Kit).

Evaluate results

The C_t values are plotted against the log of the number of cells in the lysis reaction. The resulting line is linear for cell numbers that are compatible with the procedure and deviates from linearity at concentrations that result in incomplete lysis or RT-PCR inhibition.

Xeno control

The cDNA can be amplified in parallel using the Xeno™ RNA PCR primers (from the SYBR™ Green Cells-to-C_T™ Control Kit). In contrast to the series of PCRs for an endogenous control gene, the number of cells in the lysis reaction have no effect on the C_t value that is seen in Xeno™ RNA amplification reactions, because each reaction contains the same amount of Xeno™ RNA target.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Item	Source
Cells-to-C_T™ Kit	
SYBR™ Green Fast Advanced Cells-to-C_T™ Kit	A35379
Instrument	
For reverse transcription, one of the following or equivalent:	
Veriti™ 96-Well Thermal Cycler	4375786
For real-time PCR, one of the following:	
QuantStudio™ 3 and 5 Real-Time PCR Systems	A31668, A31671
QuantStudio™ 6 and 7 Flex	4485697, 4485698
QuantStudio™ 12K Flex	4471050
Equipment	
Vortex mixer	MLS
Microcentrifuge	MLS
Pipets	MLS
Consumables	
Nuclease free pipette tips	MLS
Nuclease free microcentrifuge tubes	MLS
U-bottom 96-well plates (for cells not cultured in 96- or 384-well plates)	MLS
Real-time PCR tubes or multiwell plates appropriate for your instrument	MLS
Reagents	
RT-PCR grade water	MLS
PBS (1X), pH 7.4	AM9624
(Optional) SYBR™ Green Cells-to-C_T™ Control Kit	4386995

Item	Source
Software	
Primer Express™ Software	4363991

Contents and storage

Component	40 rxn (Cat. No. A35379)	100 rxn (Cat. No. A35380)	400 rxn (Cat. No. A35381)	Storage
Stop Solution	200 µL	500 µL	2 x 1.0 mL	–20°C
DNase I	22 µL	55 µL	220 µL	
20X RT Fast Advanced Enzyme Mix	110 µL	275 µL	1.1 mL	
2X Fast Advanced RT Buffer	2.2 mL	5.5 mL	22 mL	–20°C [1]
Lysis Solution	2.2 mL	5.5 mL	22 mL	4°C
PowerUp™ SYBR™ Green Master Mix	1.0 mL	5.0 mL	4 x 5.0 mL	

[1] After the RT Buffer is thawed for the first time, it can be stored at 4°C.

Procedural guidelines

- Use fresh cultured cells. When using frozen cultured cells, ensure that the cells were washed in cold PBS before freezing. Start the procedure by thawing the cells on ice and proceeding from “Prepare the Cells-to-CT lysate” on page 5.
- To reduce wasted reagent, dispense Stop Solution using a multichannel pipet from a set of strip tubes or a 96-well plate instead of a reagent reservoir.
- To avoid bubble formation when mixing, set the pipet to less than the reaction volume and expel the solution without emptying the pipet tip completely.
- Unless otherwise stated, room temperature is 20–25°C.
- Minus-RT controls contain all the RT reaction components except the 20X RT Enzyme Mix (substitute water). Minus-RT controls show that the template for the PCR was cDNA, and not genomic DNA.
- No-template controls (NTC) contain all the PCR components except the cell lysate (substitute water). If the no-template control yields a fluorescent signal, the RT or PCR reagents can be contaminated with DNA, for example, PCR product from previous reactions.
- Fluorescent contaminants can cause false-positive results in real-time PCR using SYBR Green dye, therefore it can be necessary to include a No-amplifIcation Control (NAC) that contains sample and all the PCR components except the PowerUp™ SYBR™ Green Master Mix. If the absolute fluorescence of the NAC is greater than that of the NTC after PCR, fluorescent contaminants can be present in the sample or in the heat block of the thermal cycler.

Before you begin

- Thaw the Stop Solution and mix thoroughly by inverting or flicking several times, then place on ice.

  IMPORTANT! Do not vortex.

- Chill 1X PBS to 4°C.

- *(Optional)* Add DNase I (1:100) to Lysis Solution, to remove genomic DNA during cell lysis, according to the following table.

Component	Volume		
	per reaction	96 reactions[1]	384 reactions
Lysis Solution	49.5 µL	5.23 mL	20.91 mL
DNase I	0.5 µL	52.8 µL	211 µL
Total	**50 µL**	**5.28 mL**	**21.12 mL**

[1] Includes 10% overage

- *(Optional)* To include an exogenous control using the SYBR™ Green Cells-to-C_T™ Control Kit, add 1 µL of Xeno™ RNA Control per 5 µL of Stop Solution.

Prepare primers

Prepare primers for the targets in the list for use in the following RT-PCR reactions.

- Amplify any endogenous control target, for example using the β-actin PCR primer set included in the SYBR™ Green Cells-to-C_T™ Control Kit.
- If Xeno™ RNA Control was added to samples, set up a separate PCR using the Xeno™ RNA PCR primer set included in the SYBR™ Green Cells-to-C_T™ Control Kit.
- Use PCR primers for the target of interest and evaluate real-time PCR of the cell titration, to help determine the minimum number of cells that are required for its detection.

Prepare cells for lysis

Prepare adherent or suspension cells for lysis.

Cell type	To prepare cells for lysis
Cells grown, including adherent and suspension cells	**1.** Detach adherent cells from the culture vessel, using a common subculturing technique such as trypsin. If trypsin is used, be sure to inactivate it before proceeding. **2.** Count the cells, then gently centrifuge to collect the contents at the bottom. **3.** Aspirate and discard the medium, then place the cells on ice. **4.** Wash the cells by resuspending them in 0.5 mL of 4°C PBS per 10^6 cells, then gently centrifuge to collect the contents at the bottom. **5.** Aspirate and discard as much of the PBS as possible without disturbing the pellet, then place the cells on ice. **6.** Resuspend the cells in fresh, 4°C PBS so that there are 2×10^5 cells/µL, then place on ice.

Prepare ten-fold serial dilutions

1. Prepare 5 tubes containing 45 µL of cold PBS, then place them on ice.
2. Transfer 5 µL of the 2×10^5 cells/µL to the first tube (1:10 dilution), then mix gently but thoroughly.
3. Continue making the serial dilutions by transferring 5 µL of each solution to the subsequent tube to finish with 5 suspensions containing 2×10^4, 2000, 200, 20, and 2 cells per µL.
4. In triplicate, transfer 5 µL of each cell suspension to individual reaction tubes or wells of a multiwell plate.

   The final cell counts are 10^5, 10^4, 1000, 100, and 10 cells.

Prepare the Cells-to-CT lysate

1. Add 50 µL of Lysis Solution to each sample, then mix the lysis reaction by pipetting up and down 5 times or by gently shaking on an orbital shaker.
2. Incubate the lysis reaction for 5 minutes at room temperature.

3. Mix the lysis reaction by pipetting up and down five times or by gentle shaking on an orbital shaker.

   IMPORTANT! Thoroughly mix the Stop Solution into the lysate.

4. Incubate for 2 minutes at room temperature.

Perform reverse transcription (RT)

1. In a nuclease-free microcentrifuge tube on ice, prepare an RT Master Mix for the number of reactions required plus 10% overage, according to the following table.

   Up to 45% of the RT reaction volume (22.5 µL) can be Cells-to-C_T™ lysate. Adjust the volume of Nuclease-free Water accordingly.

Component	1 reaction	96 reactions[1]	384 reactions[1]
2X Fast Advanced RT Buffer	25 µL	2.64 mL	10.56 mL
20X Fast Advanced RT Enzyme Mix[2]	2.5 µL	264 µL	1.056 mL
Nuclease-free Water	12.5 µL	1.32 mL	5.28 mL
Total	**40 µL**	**4.22 mL**	**16.9 mL**

[1] Volumes include 10% overage.

[2] For the minus-RT control, use Nuclease-free water instead of 20X Fast Advanced RT Enzyme Mix.

2. Distribute RT Master Mix to nuclease-free PCR tubes or wells of a multiwell plate.

3. Add sample lysate to each aliquot of RT Master Mix for a final 50-µL reaction volume.

4. Mix reactions gently, then centrifuge briefly to collect the contents at the bottom of the reaction container.

5. Set up the thermal cycler (or real-time PCR instrument) as indicated in the following table, then load and run the reactions.

Step	Stage	Cycles	Temperature	Time
Reverse transcription (hold)	1	1	37°C	30 minutes
RT inactivation (hold)	2	1	95°C	5 minutes
Hold	3	1	4°C	Indefinite

Perform qPCR

1. In a nuclease-free microcentrifuge tube at room temperature, prepare the PCR Cocktail plus 10% overage according to the following table.

Component	10 µL PCR reaction	20 µL PCR reaction
PowerUp™ SYBR™ Green Master Mix	5 µL	10 µL
PCR primers Forward and Reverse primers[1]	Variable	Variable
Nuclease-free Water	Variable	Variable
Total	**8 µL**	**16 µL**

[1] Recommended final concentration of each primer is 200–400 nM.

2. Distribute the PCR Cocktail into individual PCR tubes or wells of a real-time PCR plate at room temperature.

3. Set up the real-time PCR instrument as indicated in the following table, then load and run the reactions.

   Specify SYBR Green fluorescent dye for the experiment.

Step	Stage	Cycles	Temperature	Time
UDG activation	1	1	50°C	2 minutes
Enzyme activation (hold)	2	1	95°C	10 minutes
PCR	3	40	95°C	3 seconds
			60°C	30 seconds
Dissociation curve	4	Use default setting		

IMPORTANT! PowerUp™ SYBR™ Green Master Mix contains ROX™ passive reference dye.

Evaluate the results

Endogenous control

Create a chart of C_t versus the log of the number of cells in the lysis.

The C_t values decrease in a linear fashion as the number of cells increase, for cell numbers that are compatible with the procedure. When the number of cells per lysis reaction exceeds the capacity of the system, resulting in incomplete lysis or inhibition of RT-PCR, the data are not linear. In future experiments, do not exceed the number of cells per lysis reaction that provided results in the linear range in the pilot experiment.

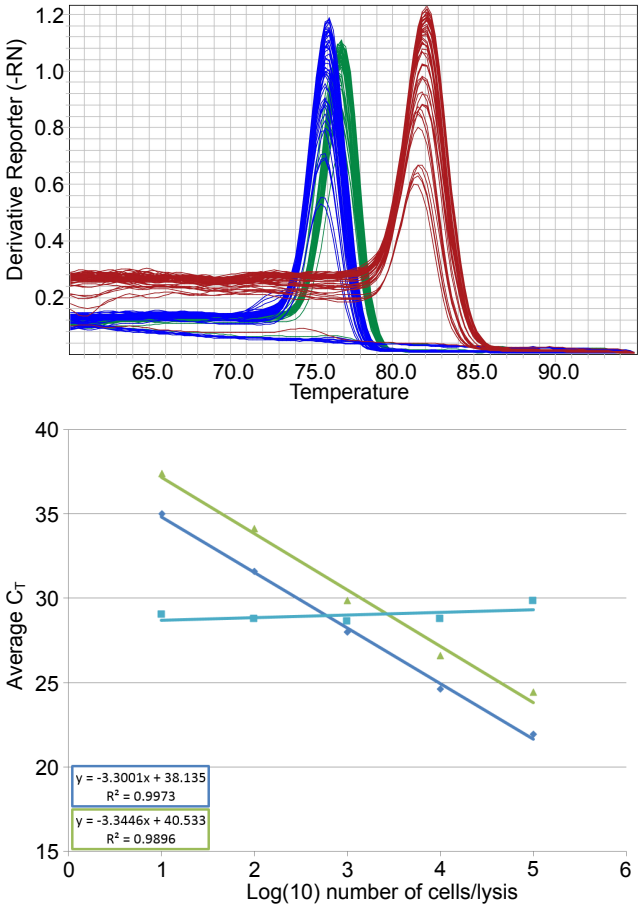

Figure 1 Real-time RT-PCR using the SYBR™ Green Fast Advanced Cells-to-C_T™ Kit

A dilution series of 10^1 to 10^5 HeLa cells was processed in triplicate with the SYBR™ Green Fast Advanced Cells-to-C_T™ Kit. The BAX and KIAA genes, and Xeno Control , were amplified from the cDNA in 10 µL reactions. The dissociation curves (top) indicate that the reactions are free of primer-dimer or any other spurious products. The red curve is BAX, blue is KIAA, and green is Xeno™ Control. The flat lines are NTCs. The standard curve (bottom) shows the threshold cycle (C_t) compared to the input number of cells for BAX. BAX is Bcl-2-associated X (dark blue diamond), KIAA is WASH Complex Subunit 4 (green triangle), Xeno is Xeno™ RNA (light blue square). Amplification was linear over a cell input range of 10^1 to 10^5 cells per lysis.

Xeno™ RNA Control

The C_t values from the Xeno™ RNA Control are consistent (±1 C_t), regardless of the number of cells in the lysis reaction, indicating that no RT-PCR inhibitors are present in the Cells-to-C_T™ lysate. Higher C_t values at higher numbers of cells per lysis reaction indicate that inhibitors were introduced into RT-PCR with this number of cells. For future experiments, use only the number of cells per lysis reaction that did not show an increase in C_t value.

Target of interest

The pilot experiment can provide useful information regarding the number of cells that are required to detect the target of interest. Examine the results carefully and select cell numbers that provide sufficient signal for the experiment.

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For descriptions of symbols on product labels or product documents, go to **thermofisher.com/symbols-definition**.

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Revision history: Pub. No. MAN0017931

Revision	Date	Description
B.0	12 May 2020	Updated topic names to have the correct MAN#. Updated the topic "Prepare five-fold serial dilutions" to be "Prepare ten-fold serial dilutions" and at step 2 from stating a 1:5 dilution to a 1:10 dilution.
A.0	14 June 2018	New document.