SingleShot Cell Lysis Kit

Catalog #	Description
1725080	**SingleShot Cell Lysis Kit**, 100 x 50 µl reactions
1725081	**SingleShot Cell Lysis Kit**, 500 x 50 µl reactions

For research purposes only.

Introduction

The SingleShot Cell Lysis Kit prepares genomic DNA (gDNA)–free RNA directly from cell culture in approximately 20 min for use in reverse transcription quantitative PCR (RT-qPCR) applications. This kit is compatible with an input of 100,000–10 cells from suspension, adherent cells, or primary cells from cell cultures. With the SingleShot Cell Lysis Kit, gene expression analysis can be completed in approximately 2 hours from cell culture to quantification cycle (Cq) using either a two-step or a one-step RT-qPCR workflow.

Kit Contents

Cell Lysis Reagents	100 x 50 µl reactions	500 x 50 µl reactions
SingleShot Cell Lysis Buffer	5 ml (1 x 5 ml vial)	25 ml (5 x 5 ml vials)
Proteinase K Solution	100 µl (1 x 1 ml vial)	500 µl (1 x 1 ml vial)
DNase Solution	100 µl (1 x 1 ml vial)	500 µl (1 x 1 ml vial)

Store all components at –20°C for up to 1 year.

RT-qPCR Products Required but Not Provided

The SingleShot Cell Lysis Kit is validated for the products listed in Table 1.

Table 1. Validated RT-qPCR products for use with SingleShot Cell Lysis Kit.

Catalog #	Description
Two-Step RT-qPCR Products	
1725038	**iScript Advanced cDNA Synthesis Kit for RT-qPCR**, 100 reactions
1725271	**SsoAdvanced Universal SYBR® Green Supermix**, 500 reactions
1725281	**SsoAdvanced Universal Probes Supermix**, 500 reactions
One-Step RT-qPCR Products	
1725151	**iTaq Universal SYBR® Green One-Step Kit**, 100 reactions
1725141	**iTaq Universal Probes One-Step Kit**, 100 reactions
One-Step Multiplex RT-qPCR Products	
12010176	**Reliance One-Step Multiplex Supermix**, 200 reactions
12010220	**Reliance One-Step Multiplex Supermix**, 1,000 reactions
12010221	**Reliance One-Step Multiplex Supermix**, 2,000 reactions
Control (optional)	
Online order only	**PrimePCR Reverse Transcription Control Assay (bio-rad.com/PrimePCR)**

Additional Reagents Required but Not Provided

- Phosphate buffered saline (PBS) for washing the cells
- TE buffer (nuclease-free), pH 7.5, for use with the PrimePCR Reverse Transcription Control Assay

Processing of Adherent Cells in a 96-Well Culture Plate

(!) For processing adherent cells in non–96-well cell culture plates, refer to Table 1 in Appendix A

(!) For processing trypsinized adherent cells, neutralize the trypsin with culture medium. Follow instructions in Processing of Nonadherent Cells in a 96-Well PCR Plate section

1. Seed the cell culture in advance in a 96-well culture plate so that the cell numbers at harvest are in the range of 100,000–10 cells/well.

   (!) For adherent cells, it is important to use cells that are fully adhered to the plate to avoid cell loss during washing

   (!) Using too many cells may result in incomplete cell lysis and can inhibit RT-qPCR. For optimal results, we recommend using the PrimePCR Reverse Transcription Control Assay to determine the appropriate input cell number to use

2. Prepare fresh on ice the appropriate volume of SingleShot cell lysis master mix (Table 2). Mix thoroughly and centrifuge. Use within 2 hr.

Table 2. Preparation of SingleShot cell lysis master mix for adherent cells.

Component	Volume per Well, µl	Volume for 96-Well Plate, µl
SingleShot Cell Lysis Buffer	48	4,608
Proteinase K Solution	1	96
DNase Solution	1	96

3. Remove cell culture medium completely by aspiration.
4. Wash cells with 125 µl of room temperature PBS. Aspirate to remove PBS completely.
5. Add 50 µl of SingleShot cell lysis master mix to each well.
6. Incubate without agitation for 10 min at room temperature.

   (!) Do not mix the cells with the solution by pipetting. For step 6, do not exceed 20 min at room temperature

7. Transfer the cell lysate to a PCR plate or centrifuge tube. Incubate at 37°C for 5 min followed by 5 min at 75°C.

   (i) Use a thermal cycler for best thermal uniformity

8. The cell lysate can be stored on ice for up to 4 hr, at –20°C for up to 2 months, or at –80°C for up to 12 months.
9. Go to the Preparation of RT-qPCR Reactions section.

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Processing of Nonadherent Cells in a 96-Well PCR Plate

1. Prepare fresh on ice the appropriate volume of SingleShot cell lysis master mix (Table 3). Mix thoroughly and centrifuge. Use within 2 hr.

Table 3. Preparation of SingleShot cell lysis master mix for nonadherent cells.

Component	Volume per Well, µl	Volume for 96-Well Plate, µl
SingleShot Cell Lysis Buffer	48	4,608
Proteinase K Solution	1	96
DNase Solution	1	96

2. Count the cells. Transfer appropriate number of cells (10–10^5 cells per well) to a 96-well PCR plate or a tube.
3. Centrifuge at 500–1,000 x g for 5 min. Remove as much of the medium as possible without disturbing the cell pellet.
4. Wash cells with 125 µl of room temperature PBS. Centrifuge at 500–1,000 x g for 5 min. Carefully remove 120 µl of the supernatant using a pipet, leaving approximately 5 µl PBS in each well.
5. Add 50 µl of SingleShot cell lysis master mix to each well. Pipet up and down five times to ensure complete resuspension of the cell pellet.
6. Incubate for 10 min at room temperature, followed by 5 min at 37°C and 5 min at 75°C.
7. The cell lysate can be stored on ice for up to 4 hr, at –20°C for up to 2 months, or at –80°C for up to 12 months.
8. Go to the Preparation of RT-qPCR Reactions section.

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Preparation of RT-qPCR Reactions

For one-step and two-step RT-qPCR reactions using cell lysate prepared with the SingleShot Cell Lysis Kit, follow the instructions in Table 4 with respect to input lysate volume. Follow the respective instructions for thermal cycling and reaction volumes in the product inserts for the reagents listed in Table 4.

Analyze the RT-qPCR data using standard methodologies.

Table 4. Input volume of SingleShot cell lysate for RT-qPCR reactions.

Two-Step RT-qPCR	Input Recommended	Input Maximum
iScript Advanced cDNA Synthesis Kit for RT-qPCR (20 µl reactions)	4 µl lysate	9 µl lysate
SsoAdvanced Universal SYBR® Green Supermix or SsoAdvanced Universal Probes Supermix (20 µl reactions)	2 µl cDNA	4 µl cDNA
One-Step RT-qPCR	**Input Recommended**	**Input Maximum**
iTaq Universal SYBR® Green One-Step Kit or iTaq Universal Probes One-Step Kit (20 µl reactions)	2 µl lysate	4 µl lysate
Reliance One-Step Multiplex Supermix (20 µl reactions)	2 µl lysate	10 µl lysate

Optimizing Input Cell Number and Input Lysate Amount

For best results, the PrimePCR Reverse Transcription Control Assay, which is sold separately, can be used to determine optimal input cell number and optimal input lysate volume.

The PrimePCR Reverse Transcription Control Assay includes a synthetic RNA template that has no homology to any known sequence and a qPCR assay (SYBR® Green or probe) specific for this RNA template.

The template RNA is shipped lyophilized. Upon resuspension, store at –80°C.

Using the PrimePCR Reverse Transcription Control Assay to Determine Optimal Input Cell Number

ⓘ To determine optimal input cell number, adherent cells must be trypsinized for accurate cell counting

1. Resuspend the RNA control template in 200 µl of nuclease-free TE buffer, pH 7.5.
2. Prepare a tenfold serial dilution of 100,000–10 cells in PBS.
3. Prepare the SingleShot cell lysis master mix according to the directions in Table 5.

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Table 5. Preparation of SingleShot cell lysis master mix containing the RNA control.

Reagent	Reagent per 4-log Tenfold Dilution Series in a 96-Well Plate, µl
SingleShot Cell Lysis Buffer	235
Proteinase K Solution	5
DNase Solution	5
RNA Control Template	5
Total volume	**250**

4. Select input lysate volumes for the RT-qPCR reactions following the recommendations in Table 4. Maintain the same lysate and cDNA volumes for all reactions in this experiment. Follow the previously described RT-qPCR reaction setup and include:
   - For one-step and two-step RT-qPCR, use 1 µl of the PrimePCR Reverse Transcription Control Assay (SYBR® Green or probe, respectively) per 20 µl RT reaction
   - If desired, gene expression targets of interest can be amplified in parallel with the RNA control assay in singleplex reactions when using SYBR® Green chemistry. For probe-based chemistry, the HEX-labeled control assay may be duplexed with a non-HEX labeled probe for a gene expression target
5. Perform data analysis according to the following guidelines:
   - RNA control: Plot the Cq values for the RNA control against the log input cell numbers used to generate the lysate (Figure 1). A constant Cq value across the input cell range indicates no RT-qPCR inhibition. A deviation of >1 Cq value indicates RT-qPCR inhibition. Input cell numbers that show such a Cq deviation should be avoided. In the example shown in Figure 1, optimal performance can be achieved with 100,000–10 input cells
   - Target gene: Plot the Cq values for the target gene against the log of the number of cells used to generate the lysate (Figure 1). A decrease in Cq value is expected as cell number increases. The decrease in Cq values should be linear for cell numbers that don't exhibit inhibitory effects. Deviation from linearity results from incomplete lysis and/or RT-qPCR inhibition

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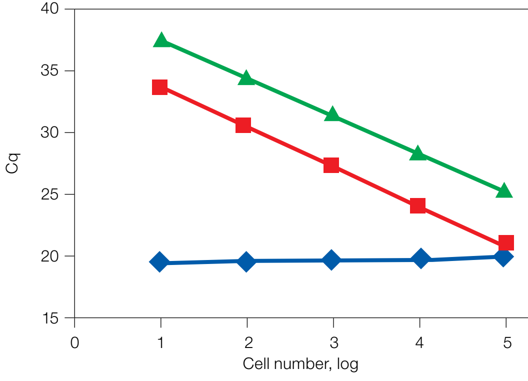

Fig. 1. Determining optimal cell input number. In this example, 10^5 input cells is the maximum input. No inhibition was noted across the input series. Target genes demonstrate linearity across all four logs. ▲, target 1; ■, target 2; ◆, control RNA. Cq, quantification cycle.

Using the PrimePCR Reverse Transcription Control Assay to Determine Optimal Input Lysate Volume

1. Resuspend the RNA control template in 200 µl of nuclease-free TE buffer, pH 7.5.
2. Prepare cell lysate from either adherent (see Processing of Adherent Cells in a 96-Well Culture Plate section) or suspension cells (see Processing of Nonadherent Cells in a 96-Well PCR Plate section) with an optimal number of input cells.
3. Vary input lysate in the RT-qPCR reactions as shown in Tables 6 and 7.
4. Program the thermal cycling protocol on a real-time PCR instrument according to the manufacturer's instructions.
5. Use 2 µl of completed RT reaction and 1 µl of the PrimePCR Reverse Transcription Control Assay in a 20 µl qPCR reaction.

Table 6. Setting up lysate titrations for two-step RT-qPCR reactions.

Two-Step RT-qPCR				
Input Lysate, %	Lysate Volume, µl	RNA Control Template, µl	2x RT Master Mix, µl*	Nuclease-Free H_2O, µl
10	2	1	10	7
20	4	1	10	5
30	6	1	10	3
40	8	1	10	1
45	9	1	10	0

* Includes 5x iScript Advanced Reaction Mix, iScript Advanced Reverse Transcriptase, and nuclease-free H_2O.

Table 7. Setting up lysate titrations for one-step RT-qPCR reactions.

One-Step RT-qPCR					
Input Lysate, %	Lysate Volume, µl	RNA Control Template, µl	RNA Control Assay, µl	2x One-Step RT-qPCR Mix, µl	Nuclease-Free H_2O, µl
10	2	1	1	10	6
15	3	1	1	10	5
20	4	1	1	10	4

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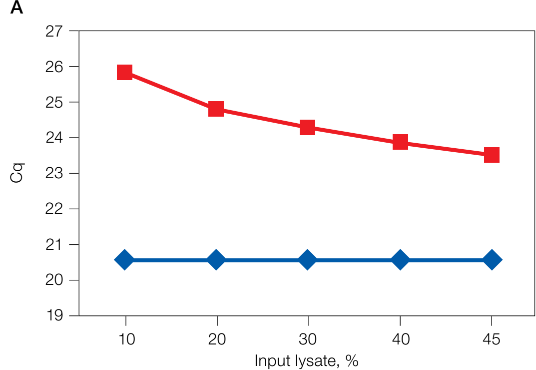
6. Perform data analysis according to the following guidelines:
   - RNA control: Plot the Cq values for the RNA control against the percentage input lysate (Figures 2A and 2B). A constant Cq value across the input lysate range indicates no RT-qPCR inhibition. A deviation of >1 Cq value indicates RT-qPCR inhibition. In the example shown in Figure 2A, optimal performance can be achieved with 100,000–10 input cells
   - Target gene: If a target gene is amplified in parallel with the RNA control, plot the Cq values for the target gene against the percentage input lysate (Figures 2A and 2B). A decrease in Cq value is expected as input lysate increases. Deviation in the linear response of the RNA control results from incomplete lysis and/or from RT-qPCR inhibition

A



B

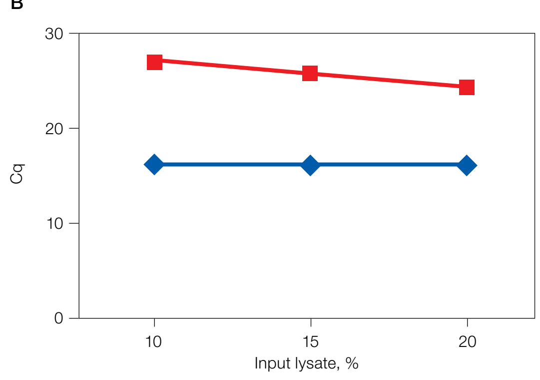


Fig. 2. Determining optimal input cell lysate amounts. A, the maximum allowable input cell lysate is 45%. **B**, this one-step RT-qPCR reaction shows no PCR inhibition across all tested input lysate amounts. ■, target; ◆, control RNA. Cq, quantification cycle.

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Appendix A

Table 1. Setting up SingleShot cell lysis reactions.

Step	Guidelines						
Preparing the cell culture	**Number of wells**	**384**	**96**	**48**	**24**	**12**	**6**
	Cell numbers per well at harvest	10– 5×10^4	10– 1×10^5	10– 2×10^5	10– 4×10^5	10– 8×10^5	10– 1×10^6
Washing the cells with PBS	Volume of PBS per well, µl	30	125	250	500	1,000	2,000
Preparing the SingleShot cell lysis master mix	SingleShot Cell Lysis Buffer, µl	12	48	96	192	384	768
	Proteinase K, µl	0.25	1	2	4	8	16
	DNase, µl	0.25	1	2	4	8	16
	Total volume per well, µl	**12.5**	**50**	**100**	**200**	**400**	**800**

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Troubleshooting Guide

Problem	Potential Cause	Solution
No amplification in the RT-qPCR reaction Delayed Cq values seen in RNA detection	▪ Cell lines may contain high levels of PCR inhibitors ▪ Excess number of cells used in the lysis reaction ▪ Excess cell culture medium carryover ▪ Excess lysate used in the RT-qPCR reaction	▪ Depending on the cell species or culture conditions, the number of cells or percentage lysate may require optimization (see Optimizing Input Cell Number and Input Lysate Amount section) ▪ Generally ≤10^5 cells can be used successfully in the SingleShot procedure, but if RT or qPCR fails, try using 5- to 10-fold fewer cells ▪ Wash cells with PBS to remove contaminants in the culture medium ▪ Remove as much of the culture medium and PBS as possible ▪ Use a freshly prepared SingleShot cell lysis master mix; keep on ice and use within 2 hr ▪ Make sure DNase and proteinase K are added to the SingleShot cell lysis master mix before treating the cells
Genomic DNA is amplified as seen in the no-RT control	▪ Incomplete gDNA digestion ▪ DNase and Proteinase K were not added to the lysis reaction	▪ Repeat the lysis step. Ensure DNase is added, the thermal cycling conditions are correct, and the thermal cycler is working properly
Signal in no template control (NTC) reaction	▪ DNA contamination from carryover, reagents, and/or water, and/or error in pipetting ▪ Primer-dimers (NTC melt peak is broad with a melting temperature ~65–75°C)	▪ Examine the workflow to identify potential contamination sources; replace reagents one by one until the contamination source is identified. Be sure to use filtered pipet tips ▪ Evaluate the assay design for primer-dimer formation; use gradient PCR to optimize the annealing temperature; use a primer matrix to determine the optimal primer concentration

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