# SingleShot™ Probes Kit

| Catalog # | Description |
|---|---|
| 172-5090 | **SingleShot Probes Kit**, 100 x 50 µl reactions |

**For research purposes only.**

## Introduction

The SingleShot Probes 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 Probes Kit, gene expression analysis can be completed in approximately 2 hours from cell culture to quantification cycle (Cq). This kit includes reagents for probe-based RT-qPCR reactions and a probe-based qPCR control assay to optimize input cell number and input lysate amount.

## Kit Contents (100 reactions)

| Cell Lysis Reagents | Description |
|---|---|
| SingleShot Cell Lysis Buffer | 5 ml (1 x 5 ml vial) |
| Proteinase K Solution | 100 µl (1 x 1 ml vial) |
| DNase Solution | 100 µl (1 x 1 ml vial) |

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

| Controls | |
|---|---|
| SingleShot RNA Control Assay | 200 reactions |
| SingleShot RNA Control Template | Lyophilized |
| SingleShot Probes qPCR Control Assay | HEX labeled, 200 µl |

Store the control template, once resuspended, at –80°C. Store the qPCR assay at –20°C for up to 1 year.

| RT-qPCR Reagents | |
|---|---|
| iScript™ Advanced cDNA Synthesis Kit for RT-qPCR | 100 reactions |
| 5x iScript Advanced Reaction Mix | 1 vial |
| iScript Advanced Reverse Transcriptase | 1 vial |
| Nuclease-Free Water | 1.5 ml |
| SsoAdvanced™ Universal Probes Supermix | 5 x 1 ml vials (500 reactions) |

Store the RT-qPCR products at –20°C for up to 1 year. The SsoAdvanced Supermix can be stored at 4°C for up to 3 months.

### Reagents Required but Not Provided

- Phosphate buffered saline (PBS) for washing the cells
- TE buffer (nuclease-free) pH 7.5 for resuspending the SingleShot RNA control template

## 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 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 SingleShot RNA control, included in this kit, to determine the appropriate input cell number

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

**Table 1. Preparation of SingleShot cell lysis master mix for adherent cells.**

| Component | Volume per Well, µl | Volume for 96 Reactions, 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 for up to 4 hr on ice, for up to 2 months at –20°C, or for up to 12 months at –80°C.
9. Go to the Preparation of Reverse Transcription Reactions section.

## 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 2). Mix thoroughly and centrifuge. Use within 2 hr.

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

| Component | Volume per Well, µl | Volume for 96 Reactions, 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 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 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 5 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 Reverse Transcription Reactions section.

## Preparation of Reverse Transcription Reactions

1. For optimal results, reactions should be assembled on ice. Prepare the reverse transcription reaction according to the directions in Table 3. Mix thoroughly by pipetting up and down several times.

**Table 3. Preparation of reverse transcription reaction.**

| Component | Volume per Reaction, µl |
|---|---|
| 5x iScript Advanced Reaction Mix | 4 |
| iScript Advanced Reverse Transcriptase | 1 |
| Cell Lysate | 4–9 |
| Nuclease-Free Water | Variable |
| **Total volume** | **20** |

2. Incubate the complete reaction mix in a thermal cycler using the following protocol: reverse transcription for 30 min at 42°C followed by RT inactivation for 5 min at 85°C.
3. Proceed to the Preparation of qPCR Reactions section or store the cDNA at –20°C.

ⓘ Recommendations for the use of no-RT control

- Interference of gene expression analysis by gDNA carryover in cell lysate samples can be tested by setting up a no-RT control reaction
- The reverse transcriptase volume in a no-RT control reaction should be replaced with water
- The same amount of cell lysate used in the RT reaction should be used in the no-RT reaction to ensure similar carryover of cDNA synthesis components in a qPCR reaction

## Preparation of qPCR Reactions

### Instrument Compatibility

SsoAdvanced Universal Probes Supermix is compatible with all Bio-Rad and other commercially available real-time PCR systems.

### Reaction Mix Preparation and Thermal Cycling Protocol

1. Thaw SsoAdvanced Universal Probes Supermix and other frozen reaction components to room temperature. Mix thoroughly, centrifuge briefly to collect solution at the bottom of tubes, then store on ice protected from light.
2. Prepare (on ice or at room temperature) enough qPCR reaction mix for all qPCR reactions by adding all required components, except the template, according to the recommendations in Table 4.

**Table 4. qPCR reaction setup.***

| Component | Volume per 10 µl Reaction, µl | Volume per 20 Reactions, µl | Final Concentration |
|---|---|---|---|
| SsoAdvanced Universal Probes Supermix (2x) | 5 | 10 | 1x |
| Forward and Reverse Primers | Variable | Variable | 250–900 nM** each primer |
| Fluorogenic Probe | Variable | Variable | 150–250 nM each probe |
| cDNA (**add at step 4**) | 1–2 | 2–4 | — |
| Nuclease-Free Water | Variable | Variable | — |
| **Total reaction mix volume** | **10** | **20** | **—** |

* Scale all components proportionally according to sample number and reaction volumes.

** For duplex assays with large ΔCq ($\Delta C_T$) values, decreasing the primer concentrations for the higher-expressing target may help. To validate, perform a primer matrix to determine optimal final primer concentration. Cq, quantification cycle; $C_T$, threshold cycle.

3. Mix the qPCR reaction mix thoroughly to ensure homogeneity and dispense equal aliquots into each PCR tube or into the wells of a PCR plate. Use good pipetting technique to ensure assay precision and accuracy.
4. Add cDNA (prepared in the Preparation of Reverse Transcription Reactions section) to the PCR tubes or wells containing qPCR reaction mix (prepared using Table 4), seal tubes or wells with flat caps or optically transparent film, and vortex 30 sec or more to ensure thorough mixing of the reaction components. Spin the tubes or plate to remove any air bubbles and to collect the reaction mixture in the vessel bottom.
5. Program the thermal cycling protocol on a real-time PCR instrument according to Table 5.

**Table 5. Thermal cycling protocol.**

| Real-Time PCR System | Setting/ Scan Mode | Polymerase Activation and DNA Denaturation | Amplification | | |
|---|---|---|---|---|---|
| | | | Denaturation at 95°C, sec | Annealing/ Extension and Plate Read at 60°C, sec* | Cycles |
| Bio-Rad® CFX96™, CFX384™, CFX96 Touch™, CFX96 Touch Deep Well, CFX384 Touch™, CFX Connect™ | All channels | 30 sec at 95°C for cDNA | 5–15 | 10–30 | 35–40 |
| Bio-Rad® iQ™5, MiniOpticon™, Chromo4™, MyiQ™ | Standard | | | 15–30 | |
| Applied Biosystems 7500 and 7900 HT, QuantStudio, StepOne, StepOnePlus, ViiA 7 | Fast | | | 10–30 | |
| | Standard | | | 60 | |
| Roche LightCycler 96 or 480 | Fast | | | 10–30 | |
| | Standard | | | 60 | |
| QIAGEN Rotor-Gene and Stratagene Mx series | Fast | | | 10–30 | |

* Shorter annealing/extension times (1–10 sec) can be used for amplicons <100 bp. Longer annealing/ extension times (30–60 sec or more) can be used for amplicons >250 bp, GC- or AT- rich targets, low-expressing targets, crude samples, or higher input amounts (for example, 4 µl of cDNA).

6. Load the PCR tubes or plate into the real-time PCR instrument and start the PCR run.
7. Perform data analysis according to the instrument-specific instructions.

ⓘ Recommendations for Assay Design and Optimization

- For best qPCR efficiency, design assays targeting an amplicon size of 70–150 bp
- The SsoAdvanced Universal Probes Supermix and the recommended qPCR cycling protocols have been optimized for assays with a primer melting temperature ($T_m$) of 60°C designed using the open source Primer3, Primer3Plus, or Primer-BLAST programs at their default settings. If primers are designed using other programs, adjust the annealing temperature accordingly
- The probe's $T_m$ should be 8–10°C higher than the calculated primer $T_m$
- In a duplex reaction, for best results apply the brighter fluorophores to the lower-expressing targets and the dimmer fluorophores to the higher-expressing targets

## Optimizing Input Cell Number and Input Lysate Amount

For best results, the SingleShot RNA control can be used to determine optimal input cell number and optimal input lysate volume.

The SingleShot RNA control includes a synthetic RNA template that has no homology to any known sequence and a qPCR assay specific for this RNA template.

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

### Using the SingleShot RNA Control 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 6.

**Table 6. 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. Perform the reverse transcription reactions following the previously mentioned protocol.
5. Prepare the qPCR reactions following the recommendations in Table 7. Do not add cDNA until step 8. Maintain the same input lysate, cDNA and qPCR volumes for all reactions in this experiment.

   ⓘ If desired, gene expression targets of interest with non-HEX probes can be amplified in parallel with the RNA control assay. Adjust volumes in Table 7 accordingly

**Table 7. Preparation of qPCR reaction mix for RNA control assay.***

| Component | Volume per 10 µl Reaction, µl | Volume per 20 µl Reactions, µl | Final Concentration |
|---|---|---|---|
| SsoAdvanced Universal Probes Supermix (2x) | 5 | 10 | 1x |
| SingleShot™ Probes qPCR Control Assay | 0.5 | 1 | 1x |
| cDNA (**add at step 8**) | 1–2 | 2–4 | — |
| Nuclease-Free water | Variable | Variable | — |
| **Total reaction mix volume** | **10** | **20** | **—** |

* Scale all components proportionally according to sample number and reaction volumes.

6. Mix the qPCR reaction mix thoroughly to ensure homogeneity and dispense equal aliquots into each PCR tube or into the wells of a PCR plate. Use good pipetting technique to ensure assay precision and accuracy.
7. Add cDNA to the PCR tubes or wells containing qPCR reaction mix (prepared using Table 7), seal tubes or wells with flat caps or optically transparent film, and gently vortex to ensure thorough mixing of the reaction components. Spin the tubes or plate to remove any air bubbles and to collect the reaction mixture in the vessel bottom.
8. Program the thermal cycling protocol on a real-time PCR instrument according to Table 5.

9. Perform data analysis according to the following guidelines:

   - RNA control: Plot the Cq values for the RNA control against the log of the number of cells used to generate the lysate (Figure 1). A constant Cq value across the input cell range indicates complete lysis and no RT-qPCR inhibition. A deviation of >1 Cq value indicates incomplete lysis and/or 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. A deviation from linearity results from incomplete lysis and/or RT-qPCR inhibition.

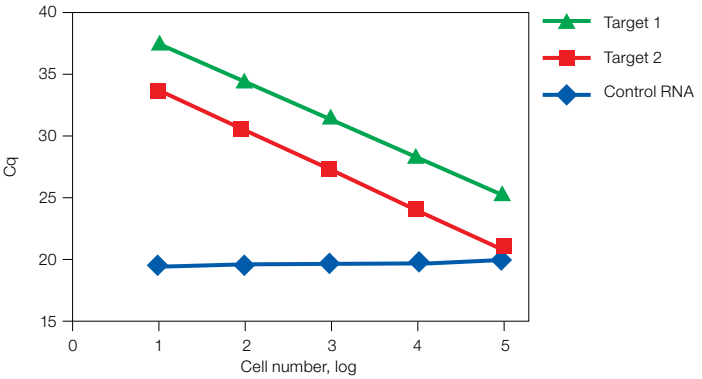


**Fig. 1. Determining optimal cell input number. In this example, $10^5$ input cells is the maximum input.** No inhibition was noted across the input series. Target genes demonstrate linearity across all four logs.

**Using the SingleShot RNA Control 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 cell lysate in the reverse transcription reactions as shown in Table 8.

**Table 8. Setup of reverse transcription reaction to optimize input cell lysate amount.**

| 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 $H_2O$.

4. Incubate the complete reaction mix in a thermal cycler using the following protocol: reverse transcription for 30 min at 42°C followed by RT inactivation for 5 min at 85°C.
5. Set up qPCR reactions following instructions in Table 9. Do not add cDNA until step 7.

**Table 9. Preparation of qPCR reaction mix for RNA control assay.***

| Component | Volume per 10 µl Reaction, µl | Volume per 20 µl Reactions, µl | Final Concentration |
|---|---|---|---|
| SsoAdvanced Universal Probes Supermix (2x) | 5 | 10 | 1x |
| SingleShot™ Probes qPCR Control Assay | 0.5 | 1 | 1x |
| cDNA (**add at step 7**) | 1–2 | 2–4 | — |
| Nuclease-Free Water | Variable | Variable | — |
| **Total reaction mix volume** | **10** | **20** | **—** |

* Scale all components proportionally according to sample number and reaction volumes.

6. Mix the qPCR reaction mix thoroughly to ensure homogeneity and dispense equal aliquots into each PCR tube or into the wells of a PCR plate. Use good pipetting technique to ensure assay precision and accuracy.
7. Add cDNA to the PCR tubes or wells containing qPCR reaction mix (prepared using Table 9), seal tubes or wells with flat caps or optically transparent film, and gently vortex to ensure thorough mixing of the reaction components. Spin the tubes or plate to remove any air bubbles and to collect the reaction mixture in the vessel bottom.
8. Program the thermal cycling protocol on a real-time PCR instrument according to Table 5.

9. Perform data analysis according to the following guidelines:
   - RNA control: Plot the Cq values for the RNA control against the percentage input lysate (Figure 2). A constant Cq value across the input lysate range indicates no RT-qPCR inhibition. A deviation of >1 Cq value indicates RT-qPCR inhibition. Input lysate amounts that show such a Cq deviation should be avoided. In the example shown in Figure 2, optimal performance can be achieved with input lysate amounts up to 45%.
   - 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 (Figure 2). A decrease in Cq value is expected as input lysate increases. A deviation in the linear response of the RNA control results from incomplete lysis, and/or from RT-qPCR inhibition.

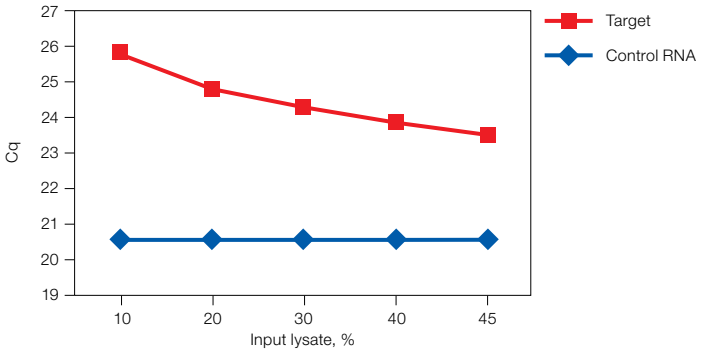


**Fig. 2. Determining optimal input cell lysate amounts.** The maximum allowable cell lysate input is 45%.

## 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 x $10^4$ | 10–1 x $10^5$ | 10–2 x $10^5$ | 10–4 x $10^5$ | 10–8 x $10^5$ | 10–1 x $10^6$ |
| 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** |

* Prepare excess SingleShot cell lysis master mix to ensure enough reagent is available.

## Troubleshooting Guide

| Problem | Potential Cause | Solution |
|---|---|---|
| No amplification in the RT-qPCR reaction<br>Delayed Cq values seen in RNA detection | ▪ Cell lines may contain high levels of PCR inhibitors<br>▪ Excess number of cells used in the lysis reaction<br>▪ Excess cell culture medium carryover<br>▪ Excess lysate used in the RT-qPCR reaction | ▪ Depending on the cell type or culture conditions, the input number of cells or percentage lysate may require optimization (see Optimizing Input Cell Number and Input Lysate Amount section)<br>▪ 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<br>▪ Wash cells twice with PBS to minimize inhibition from excess cell culture medium carryover<br>▪ Remove as much of the culture medium and PBS as possible<br>▪ Use a freshly prepared SingleShot cell lysis master mix; keep on ice and use within 2 hr<br>▪ Make sure DNase and proteinase K are added in the SingleShot cell lysis master mix before cell lysis |
| Genomic DNA is amplified as seen in the no-RT control | ▪ Incomplete gDNA digestion<br>▪ 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 (NTC melt peak $T_m$ is identical to the target gene melt peak $T_m$)<br>▪ Primer dimers (NTC melt peak is broad with a $T_m$ ~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<br>▪ 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. |

## Visit bio-rad.com/web/SSProbes for more information.



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