iScript Explore One-Step RT and PreAmp Kit

Catalog #	Description
12004856	**iScript Explore One-Step RT and PreAmp Kit**, 50 x 50 µl reactions
17002826	**iScript Explore One-Step RT and PreAmp Kit**, 250 x 50 µl reactions

For research purposes only.

Introduction

The iScript Explore One-Step RT and PreAmp Kit enables the simultaneous reverse transcription (RT) of RNA and target-specific preamplification of up to 100 targets for unbiased synthesis of cDNA. The kit contains all the materials necessary to remove residual genomic DNA (gDNA) from purified RNA samples and reverse transcribe and preamplify targets of interest; just add RNA sample and PreAmp Assay primers into the reaction to create enriched cDNA ready for quantitative PCR (qPCR).

Storage and Stability

Upon receipt, store the contents of the kit according to Table 1. Kit contents are guaranteed to be stable for 12 months if stored under the recommended conditions.

Table 1. Kit contents and storage recommendations.

Reagent	Description	Storage Temperature, ˚C
iScript DNase Enzyme	Concentrated custom DNase I solution	–20
iScript DNase Buffer	Concentrated proprietary DNase buffer solution	4
iScript Advanced Reverse Transcriptase	RNase H+ Moloney murine leukemia virus (MMLV) RT enzyme and RNase inhibitors	–20
iScript Explore Reaction Booster	Proprietary mixture of preamplification reaction enhancers	–20
SsoAdvanced PreAmp Supermix	Preamplification reaction master mix optimized for unbiased target-specific preamplification	–20
Nuclease-Free Water	RNase-/DNase-free purified water	4

Review Before You Begin

- The procedure will also require standard molecular biology consumables, such as pipets, tips, tubes and caps, TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), and additional nuclease-free water
- The input RNA amount should be optimized based on target gene abundance and sample availability. Input RNA sample up to 1 µg may be used in the reaction. Alternatively, cell lysates generated using SingleShot Cell Lysis RT-qPCR Kits can also be used as input sample. Sample can be added to the reaction up to a maximum volume of 10.5 µl
- Refer to the PrimePCR manual (**bio-rad.com/PrimePCRmanual**) for information about PrimePCR Control Assays, their compatibility with qPCR workflows, and the interpretation of their results
- If using PrimePCR Arrays, note that the RNA template for the PrimePCR Reverse Transcription Control is provided with the array. If running this control, be sure to include it in the iScript Explore reaction as directed in the protocol

Step 1: Prepare PreAmp Assay Pool

Prepare the PreAmp Assay pool at sufficient quantities for reactions according to the following directions. Each reaction in this kit will use 5 µl of the PreAmp Assay pool. Assay pools are stable at 4°C for up to 30 days or at –20°C for up to 1 year.

Option A: Preblended PrimePCR lncRNA PreAmp Assay Pools

No preparation required. The preblended pool is already prepared at the correct molarity of primers blended into a single mix. Proceed to Step 2.

Option B: Custom PrimePCR PreAmp Assay Pools

A custom PreAmp Assay pool can be created from PrimePCR PreAmp Assays as follows:

a. Transfer 5 µl of each PrimePCR PreAmp Assay (up to 100 assays) into a new microcentrifuge tube. Add nuclease-free water, if necessary, to bring the total volume of the assay pool up to 500 µl.

b. Mix and centrifuge well.

c. Proceed to Step 2.

Option C: Self-Designed Assay Primers

If using self-designed assays, a custom PreAmp Assay pool can be created using forward and reverse primers for all assays, as follows:

a. Dilute all primers to 200 µM or specify this concentration from your custom oligo provider.

b. Transfer 2.5 µl of each primer (up to 100 assays; 200 primers total) into a microcentrifuge tube. Add nuclease-free water, if necessary, to bring the volume up to 500 µl.

c. Mix and centrifuge well.

d. Proceed to Step 2.

Step 2: Thaw Reagents

2.1 Completely thaw the iScript Explore Kit components on ice.

a. Once thawed, vortex all reagents and centrifuge briefly to collect the solutions at the bottom of the tube. Store tubes on ice while preparing reactions.

b. For optimal results, reactions should be assembled on ice using nuclease-free tubes, tube strips, or plates with a 0.2 ml volume.

2.2 (Optional) If using the PrimePCR Reverse Transcription Control Assay in downstream qPCR reactions or with PrimePCR Arrays, prepare the lyophilized RNA template provided.

Resuspend the control template by adding 200 µl nuclease-free TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) to the tube and mix well. Keep template on ice as it is RNA and will degrade similarly to RNA samples if exposed to multiple freeze-thaw cycles, ambient temperatures, or RNases.

Step 3: gDNA Clearance Reaction (optional)

Note: The gDNA clearance reaction is recommended but may be omitted from the procedure if desired. If omitting this step, adjust reaction volumes accordingly by adding equivalent volumes of nuclease-free water to the one-step RT and preamplification reaction.

3.1 Prepare the DNase reaction mix by combining only the DNase enzyme and DNase buffer according to the guidelines in Table 2. If multiple reactions are required, scale up appropriately. Mix thoroughly by pipetting up and down several times.

Table 2. Setup for DNase reaction mix.

Component	Volume per Reaction, µl
iScript DNase I (blue tube)	0.5
iScript DNase Buffer (yellow tube)	1.5
Total volume	**2**

3.2 Dilute RNA samples with nuclease-free water such that the final input RNA is equivalent between samples. Refer to Table 3 for input volumes.

3.3 In a new tube, prepare the gDNA clearance reaction according to Table 3. **Important!** If using the optional PrimePCR Reverse Transcription Control Assay in downstream qPCR reactions, the RNA template must be added at this step.

Table 3. Setup for gDNA clearance reaction.

Component	Volume with RT Control, µl	Volume without RT Control, µl
Sample	10.5	11.5
DNase Reaction Mix	1.5	1.5
PrimePCR Reverse Transcription Control Template	1	–
Total volume	**13**	**13**

3.4 Pipet solution up and down to mix well. Pulse centrifuge to collect the contents at the bottom of the tube.

3.5 Using a thermal cycler with heated lid, incubate the reaction tubes according to the guidelines in Table 4.

Table 4. DNase reaction protocol.

Steps	Temperature, °C	Time Duration
DNA digestion	25	5 min
DNase inactivation	75	5 min
Storage conditions	4, on ice	Until one-step RT and preamplification reaction

Note: To avoid degradation of RNA, we recommend you proceed directly to the one-step RT and preamplification reaction. However, if longer storage is necessary, samples can be stored at –20°C and thawed on ice before proceeding to the one-step RT and preamplification reaction step.

Step 4: One-Step RT and Preamplification Reaction

4.1 Set up for the one-step RT and preamplification reaction per Table 5 and mix well. Reactions should be assembled on ice. If multiple reactions are required, scale up appropriately.

Table 5. One-step RT and preamplification reaction mix setup.

Component	Volume per Reaction, µl*
SsoAdvanced PreAmp Supermix (2x, purple tube)	25
PreAmp Assay Pool	5
Nuclease-Free Water (clear tube)	5
iScript Advanced Reverse Transcriptase (orange tube)	1
iScript Explore Reaction Booster (red tube)	1
Total volume	**37**

* The volumes in Table 5 represent the precise amount required per reaction. We recommend preparing 10% excess to account for loss during pipetting.

4.2 Add 37 µl of the one-step RT and preamplification reaction mix to each PCR tube containing 13 µl of DNase-treated RNA sample for a total reaction volume of 50 µl.

4.3 Pipet up and down to mix well. Pulse centrifuge to collect the contents at the bottom of the tube.

4.4 Program the thermal cycling protocol on a PCR instrument according to Table 6. We suggest running 14 cycles of preamplification as a starting point for target enrichment; however, the number of preamplification cycles may be adjusted from 10 up to 16 cycles.

Table 6. One-step RT and preamplification reaction protocol.

Step	Temperature, °C	Time	Cycles
Priming	25	5 min	
Reverse transcription	45	60 min	1
RT inactivation	95	3 min	
Preamplification	95	15 sec	14
	58	4 min	
Hold	4	∞	–

4.5 Load the samples into the PCR instrument and start the reaction program.

4.6 After run completion, the preamplified cDNA can be stored at 4°C for up to 72 hours or at –20°C for up to 12 months.

Step 5: Dilute Sample and Prepare for qPCR Reactions

5.1 Dilute the preamplified cDNA sample with TE buffer. The preamplified reaction cDNA products should be diluted a minimum of 1:10 with TE buffer. **Note:** A larger dilution may be needed depending on the number of downstream qPCR reactions required. A 1:50 dilution will provide enough volume for 400 qPCR reactions using 2 µl of input with technical triplicates.

5.2 For optimal results, use 2 µl of the diluted reaction products per 20 µl qPCR reaction or 1 µl per 10 µl qPCR reaction for a 96-well or 384-well plate, respectively. It is recommended to use SsoAdvanced Universal SYBR® Green Supermix or SsoAdvanced Universal Probes Supermix as the qPCR master mix, depending on the detection chemistry of the assays used.

Analysis of Results

Consider the following when interpreting data from qPCR results using preamplification reactions.

- Preamplification is a powerful tool for target enrichment prior to qPCR. However, preamplification also lowers the limit for what is considered a reliable amplification event. When performing preamplification, consider the number of preamplification cycles run when determining the quantification cycle (Cq) cutoff for reliable data. For example, with 12 cycles of preamplification, the qPCR Cq values >28 cycles should be examined carefully to ensure the robustness of the data. When using SYBR® Green Assays, confirm that the postamplification melt curves produce a single melt peak corresponding to the intended product. Replicate experiments of late amplification events may be required to validate the expression results. When using preamplification protocols, these same considerations should be applied to the PrimePCR gDNA Control Assay. Its results will also appear at an earlier Cq value than without preamplification
- Table 7 displays theoretical Cq values for 1 ng of sample run for varying preamplification cycles and diluted 1:10 before going into recommended qPCR conditions (2 µl of sample per 20 µl reaction). Use this table as a guide to aid in interpreting reliable amplification events

Table 7. Theoretical ideal effect of preamplification on qPCR reactions.

Cycles of Preamplification	Number of Cycles Earlier That Target Is Detected	Anticipated Cq Limit of Single Copy Detection
10	2.034	33
12	4.034	31
14	6.034	29

- Verification of the preamplification reaction can be assessed using the PrimePCR PreAmp Control Assay, which is integrated into the iScript Explore Kit. This control assay is typically present on PrimePCR lncRNA Arrays
- After preamplification, the diluted sample is run in two separate qPCR assays assessing the amplified (PAQ1) and unamplified (PAQ2) template. Performance of preamplification is verified if the ΔCq between the PAQ1 and PAQ2 reactions is within 1 Cq value of the number of preamplification cycles performed. For example, if 14 preamplification cycles were used, the preamplification reaction was efficient if the Cq difference between PAQ1 and PAQ2 is between 13 and 15

Associated Products

- PrimePCR lncRNA PreAmp Assay Pools
- PrimePCR lncRNA Predesigned Arrays
- PrimePCR lncRNA PreAmp Assays
- PrimePCR lncRNA SYBR® Green Assays
- PrimePCR lncRNA Probe Assays
- PrimePCR lncRNA Custom Arrays
- SsoAdvanced Universal SYBR® Green Supermix (catalog #1725270)
- SsoAdvanced Universal Probes Supermix (catalog #1725280)

Quality Control

The iScript Explore One-Step RT and PreAmp Kit demonstrates efficient reverse transcription and high multiplex preamplification efficiency of target RNAs over a wide dynamic range. Stringent specifications are maintained to ensure lot-to-lot consistency.

Visit bio-rad.com/iScriptExplore for more information.

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