Oct 27, 2025

First-Strand cDNA Synthesis from Cultured Human Epithelial Cells (HNSCC)

DOI
https://dx.doi.org/10.17504/protocols.io.kqdg311n7l25/v1

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Protocol Citation: Boluwatife Afolabi 2025. First-Strand cDNA Synthesis from Cultured Human Epithelial Cells (HNSCC). **protocols.io** https://dx.doi.org/10.17504/protocols.io.kqdg311n7l25/v1

Protocol status: Working
We use this protocol and it's working well.

Created: October 26, 2025

Last Modified: October 27, 2025

Protocol Integer ID: 230820

Keywords: cDNA synthesis, epithelial cells, cell culture, TR146, HNSCC, strand cdna synthesis from cultured human epithelial cell, strand cdna synthesis, rad iscript cdna synthesis kit, epithelial cell cultures of hnscc cell line, cultured human epithelial cell, epithelial cell culture, strand complementary dna, complementary dna, total rna, gene expression analysis, hnscc cell line, cdna, qpcr, time pcr

Abstract

This protocol describes the synthesis of first-strand complementary DNA (cDNA) from total RNA extracted from epithelial cell cultures of HNSCC cell lines using the Bio-Rad iScript cDNA Synthesis Kit. The method is optimized for downstream quantitative real-time PCR (RT-qPCR) applications and gene expression analysis.

Guidelines

Notes and Best Practices

1. **RNA Handling:** Always work in an RNase-free environment. Use dedicated pipettes and filter tips for RNA work.
2. **Reaction Scaling:** The protocol can be scaled to 10 µL reactions (half volumes) if RNA is limited, though 20 µL reactions are recommended for optimal performance.
3. **Primer Design Consideration:** The iScript kit contains a blend of oligo(dT) and random hexamer primers, providing unbiased 3' and 5' coverage. Design qPCR primers accordingly.
4. **Dynamic Range:** The iScript kit supports RNA inputs from 1 pg to 1 µg, making it suitable for both high and low-abundance transcripts.
5. **No-Template Control (NTC):** In addition to no-RT controls, include a no-template control (water only) in downstream qPCR reactions to monitor for contamination.
6. **Documentation:** Record all calculations, lot numbers of reagents, and thermal cycler used for full traceability and reproducibility.

Materials

Materials and Reagents

Equipment:

- NanoDrop™ spectrophotometer (or equivalent UV-Vis spectrophotometer)
- Thermal cycler with heated lid capability
- Microcentrifuge
- Vortex mixer
- Pipettes (P2, P10, P20, P200, P1000)
- Ice bucket with ice

Consumables:

- 0.2 mL PCR tubes (thin-walled, nuclease-free)
- Nuclease-free pipette tips (filter tips recommended)
- Nuclease-free microcentrifuge tubes (1.5 mL)

Reagents:

- Bio-Rad iScript cDNA Synthesis Kit (Cat# 1708890/1708891) containing:

1. 5× iScript Reaction Mix (4 µL per 20 µL reaction)
2. iScript Reverse Transcriptase (1 µL per 20 µL reaction)

- Nuclease-free water (molecular biology grade)
- Total RNA (extracted from epithelial cells, stored at -80°C)

RNA Quantification and Quality Assessment

1 Thaw RNA samples on ice. Mix gently by pipetting or brief vortexing.

2 Measure RNA concentration using NanoDrop™ spectrophotometer.

3 Assess RNA purity by evaluating absorbance ratios (A260/A280 and A260/A230)
Note: Proceed if both ratios are within acceptable ranges

RNA Normalization

4 **Rationale:** Since cDNA concentration is difficult to accurately quantify post-synthesis, normalize the RNA input amount to ensure consistency across samples. This standardization enables reliable comparison of gene expression levels between samples.

5 **Recommended**: 500 ng total RNA per reaction (adjustable based on RNA availability: 1 pg - 1 µg range)

cDNA Synthesis Reaction Setup

6 Thaw all iScript kit components on ice. Mix gently by inverting tubes 5-10 times. Briefly centrifuge to collect contents.

7 Calculate the number of reactions needed (include 10% overage for pipetting error):
- 5× iScript Reaction Mix: **4 µL**
- iScript Reverse Transcriptase: **1 µL**
- Total per reaction: **5 µL**
- RNA Template: **Variable**, calculated from step 5
- Water (µL) = **20−5−RNA volume (µL)**

8 Mix gently by pipetting up and down 5-8 times. Avoid introducing bubbles.

9 Briefly centrifuge tubes (2-3 seconds) to collect contents at the bottom.

10 Program the thermal cycler with the following protocol:

	Step	Temperature	Duration	Purpose
	1. Priming	25°C	5 minutes	Primer annealing
	2. Reverse Transcription	46°C	20 minutes	cDNA synthesis
	3. RT Inactivation	95°C	1 minute	Enzyme inactivation
	4. Hold	4°C	∞	Storage

Thermocyler Program for iScript cDNA Synthesis

Post-Synthesis Processing

11 **Rationale for Diluting cDNA:** Diluting cDNA reduces potential PCR inhibitors from the reverse transcription reaction and extends the usable volume for multiple downstream qPCR reactions.

12 **Recommended Dilution:** 1:3 (v/v) or 1:4 (v/v) depending on RNA input amount and expected target gene abundance:
- For 500 ng RNA input: 1:3 dilution is optimal
- For lower RNA inputs (<100 ng): 1:2 dilution or use undiluted

13 **Dilution procedure** (1:3 dilution example):
- Transfer the entire 20 µL cDNA reaction to a labeled 1.5 mL microcentrifuge tube
- Add 60 µL nuclease-free water
- Mix by pipetting or gentle vortexing
- **Final volume: 80 µL**

14 Briefly centrifuge to collect contents.

Storage and Quality Control

15 Aliquot diluted cDNA into appropriate volumes based on anticipated use:
- For frequent use (within 1 week): store at **4°C**
- For long-term storage (>1 week): store at **-20°C** or **-80°C**

16 Minimize freeze-thaw cycles. For samples requiring multiple uses, prepare 10-20 µL aliquots to avoid repeated freezing and thawing.

17 Label all tubes clearly

Protocol references

1. Bio-Rad Laboratories. (2025). iScript™ cDNA Synthesis Kit Instruction Manual. Bio-Rad Laboratories, Inc.
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Acknowledgements

Special thanks to my research advisors: Dr. Mark Herzberg, Dr. Chong Wang and Dr. Karen Johnstone.