Jul 03, 2025 Version 1

Automated high-throughput RT-PCR V.1

DOI
https://dx.doi.org/10.17504/protocols.io.bp2l6dzm1vqe/v1

Alex Veith[1], Anna Shen[1], Christopher Bradfield[1]

[1]University of Wisconsin-Madison

Alex Veith
University of Wisconsin-Madison

DOI: https://dx.doi.org/10.17504/protocols.io.bp2l6dzm1vqe/v1

Protocol Citation: Alex Veith, Anna Shen, Christopher Bradfield 2025. Automated high-throughput RT-PCR. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bp2l6dzm1vqe/v1

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

Created: February 12, 2025

Last Modified: July 03, 2025

Protocol Integer ID: 120124

Keywords: RT-PCR, Automated liquid handling, EpMotion, pcr this protocol, pcr, gene expression target, diluted rna, µl of diluted rna, different numbers of gene expression target, well pcr plate for efficiency, gene expression, rna, well pcr plate, custom rt, throughput rt, including primer, gene, technical replicate, primer, rt, step master mix

Funders Acknowledgements:
National Institute of Environmental Health Sciences
Grant ID: R35ES028377

Abstract

This protocol provides an outline for planning, setting up, and running an epMotion® M5073 for high-throughput RT-PCR. This protocol leverages a set of pre-written template programs to accommodate different numbers of gene expression targets. The template programs have been designed to dispense 2 µL of diluted RNA and 8 µL of one-step master mix (including primers and probes) to create 10 µL reactions on a 384-well PCR plate. Diluted RNA is to be arranged in a 96-well PCR plate for efficiency. We also provide the steps necessary to create custom RT-PCR runs for more advanced users. These steps also detail the settings used in our predefined programs. Users can expect consistent results with minimal variability between technical replicates that are quick and reliable.

Attachments

1 Target Large Sampl... 755KB
2 Target Large Sampl... 749KB
3 Target Large Sampl... 747KB
4 Target Large Sampl... 747KB
2 Target Simulation ... 765KB
3 Target Simulation ... 763KB
4 Target Simulation ... 762KB
5 Target Simulation ... 748KB
6 Target Simulation ... 763KB
7 Target Simulation ... 754KB
8 Target Simulation ... 765KB

Guidelines

The template protocols were optimized using RNA diluted in water (50 ng/µL), Taqman™ Gene Expression Assays, and Promega GoTaq® Probe One-Step Master Mix, and then run on a QuantStudio™. Templates must be edited if changes are made to consumables, reagents, or the protocol. Please also validate the data conversion template. Post-run log files can also be obtained with detailed run information.

Materials

Consumables

Eppendorf twin.tec® PCR Plates **Eppendorf Catalog #** 951020745 (any color)
Microcentrifuge tubes **Eppendorf Catalog #**022364111
96 Well PCR Plates, Semi-Skirted **Phenix Research Catalog #**1147B52

Only if using multichannel master mix dispensing

epMotion Reservoir 10 mL **Eppendorf Catalog #**0030126521 (may need other sizes)

Reagents

Diluted RNA (we use 50ng/µL)

GoTaq® Probe RT-qPCR Kit **Promega Catalog #**A6121 (or equivalent)
TaqMan™ Gene Expression Assay **Thermo Fisher Scientific**

Protocol materials

GoTaq® Probe RT-qPCR Kit **Promega Catalog #**A6121
TaqMan™ Gene Expression Assay **Thermo Fisher Scientific**
Eppendorf twin.tec® PCR Plates **Eppendorf Catalog #** 951020745
Microcentrifuge tubes **Eppendorf Catalog #**022364111
96 Well PCR Plates, Semi-Skirted **Phenix Research Catalog #**1147B52
epMotion Reservoir 10 mL **Eppendorf Catalog #**0030126521
epMotion® reservoir 10mL **Eppendorf Catalog #**0030126521

Safety warnings

The epMotion® does have optical sensing. However, this feature is not used in any volume detection steps. Therefore, you must be accurate and precise with your volumes.

Before start

Screenshots and equipment notes have been embedded into the notes for several steps.

1 Select the protocol depending on whether the experiment can utilize a pre-defined template program (recommended) or whether a custom program is needed. This protocol provides a basic outline for creating a custom program; however, additional training may be required.

STEP CASE

Predefined RT-PCR Template Programs 38 steps

Follow this protocol to use the predefined template programs. This is recommended for most users.

Simulation runs for each template program are attached.

Experimental Design

2 Each epBlue® protocol has been written based on the number of gene expression targets per plate (column A). Based on the number of probes and technical duplication, we have also listed the maximum number of samples that can be included on a single 384-well RT-PCR plate.

	# of Probes	Maximum Sample #
	1	192
	2	96
	3	64
	4	48
	5	32
	6	32
	7	24
	8	24

3 The pre-designed protocols have been written to perform one-step RT-PCR reactions. In these protocols, 2µL diluted RNA is mixed with 8µL master mix containing all other required reaction components for a 10µL reaction.

4 ***Estimate* Minimum RNA Volume**

The 96 Well PCR Plates, Semi-Skirted **Phenix Research Catalog #**1147B52 have a "dead volume" of 8µL with the epMotion®. Use this formula to estimate the volume of RNA required for your experiment. RNA concentration is up to the user.

Estimated Minimum RNA Volume Required = (volume RNA/well [**2**] * technical replicates [**2**] * **#** of gene expression assays) + **8**µL

Note

The estimate for the RNA volume is reliable, but it will need to be verified on the epBlue® software.

5 RNA samples will be aliquoted into an 96 Well PCR Plates, Semi-Skirted **Phenix Research Catalog #**1147B52 according to the layout below (Figure 1). Aliquot the volumes determined by the prior calculation. Each well will likely have an equal volume. The layout of the 384-well reaction plate is found at go to step #35 .

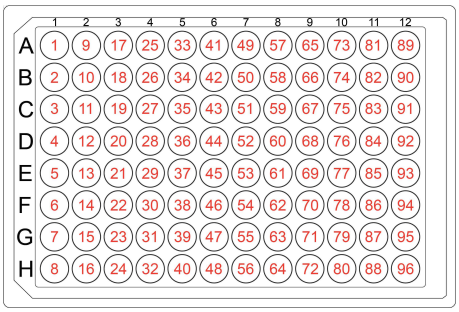


Figure 1. Plate layout.
Order RNA samples in ascending order as indicated by this layout.

Note

Ensure diluted RNA is centrifuged to eliminate splatter and bubbles

6 Select protocol format based on the user's experimental design. The default works well for low to moderate sample sizes (8-48), while the high sample format works better for experiments with a high N (>48) and a low number of gene expression targets (1-4).

STEP CASE

Single-channel Master Mix Protocols 33 steps

This is for protocols utilizing the single-channel pipette head to dispense the master mix. This is the default and is useful for multiple gene expression targets (2-8) and small sample sizes.

7 ***Estimate* Minimum Master Mix Volume** (per gene expression target)
The Microcentrifuge tubes **Eppendorf Catalog #**022364111 have a "dead volume" of 4µL. Use this formula to estimate the volume of Master Mix required for your experiment. This is the volume needed for *each* gene expression assay.

epMotion® Volume: (number of samples * volume/well [**8**] * technical replicates [**2**]) + **4**µL
Estimated Minimum Volume Required = epMotion® Volume + (**0.1** * epMotion® Volume)

Note

The estimate for the Master Mix volume is reliable, but it will need to be verified on the epBlue® software.

Sample and Master Mix Prep

8 Aliquot RNA with the minimum calculated volume into
96 Well PCR Plates, Semi-Skirted **Phenix Research Catalog #**1147B52 per the format described in Figure 1 go to step #6 .

Note

Excess volume can be prepared if desired. We often aliquot excess so that one RNA plate can be reused for multiple gene expression experiments. We have also stored RNA in sealed plates at -80°C. Regardless of the volume aliquoted, it is necessary to be accurate and record the volume for the epBlue® software.

9 Prepare Master Mix(s) for the number of gene expression assays used in this experiment (1-8).

epBlue® Login and Program Selection

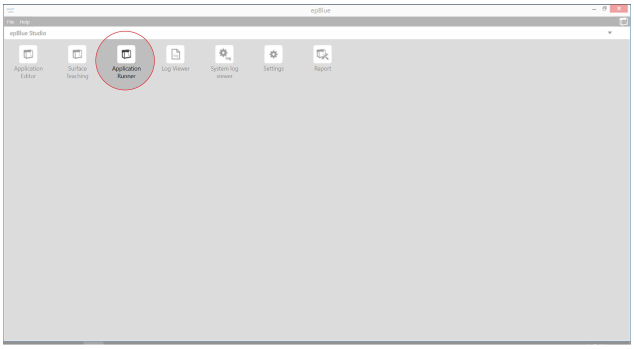
10 Log in to the epBlue® software using the guest login

user: RT_PCR_Guest
pass: guest

11 Click "Application Runner."

Note

Screenshot



12 Select the program that corresponds to the number of gene expression targets to be run on a single 384-well plate.

Note

Screenshot

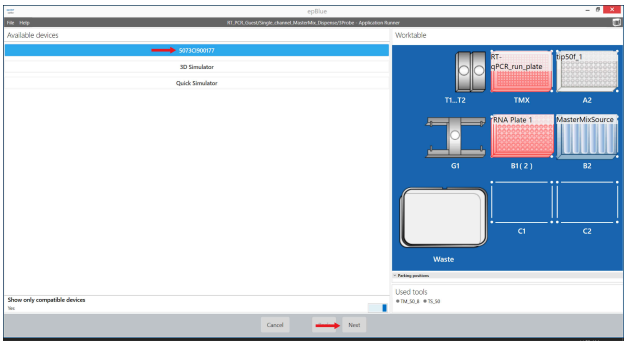
Note

The programs listed here use a single-channel pipette head to dispense master mix. A separate folder for programs uses a multichannel pipette head to dispense master mix. These programs are only recommended if you have a large number of samples (>48). The larger number of samples limits the number of targets that can be included on a single plate. go to step #6 and select Large Sample N if your experimental design requires.

13 Users are first prompted to start either a simulation or an actual run. Ensure the machine ID is selected (blue) to begin a live run. This page details the worktable layout and the tools needed for this protocol (single and multi-channel P50 pipette heads). Then click the next button to proceed.

Note

Screenshot

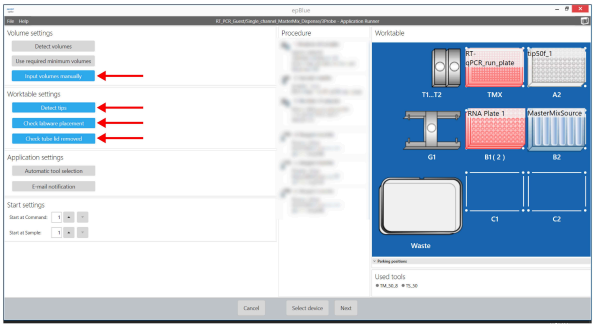


14 Users are then prompted to set up other basic parameters for each run. Please ensure that the following settings (red arrows) are selected (blue).

- Input volumes manually
- Detect tips
- Check labware placement
- Check tube lid removed

Note

Screenshot



15 Input the number of samples. The program will have a maximum number.

16 The next page shows the worktable set up. This is the same for every program, and it will be described in a future step. Click Next at the bottom of the screen.

RNA Volume Validation

17 Verify diluted RNA volume. The program will give you the minimum volume required for the run (red arrow). As each well will likely have equal volumes, select the "Set all volumes" button to quickly assign a volume to each well. This will include volumes in unused wells, but this is irrelevant.

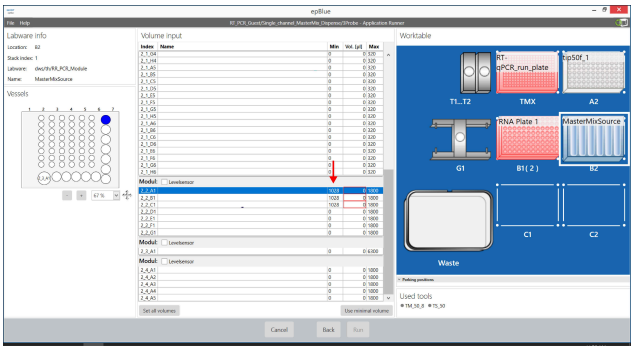
18 Input diluted RNA volumes. **Volumes must be accurate.** Click next to proceed.

Master Mix Volume Validation

19 The next page indicates the master mix volume required for each gene expression assay. The software indicates the expected amount of master mix (red arrow). In our experience, this protocol runs optimally when **at least 10%** extra master mix is prepared and is reflected in the estimate formula (go to step #7)

Note

Screenshot



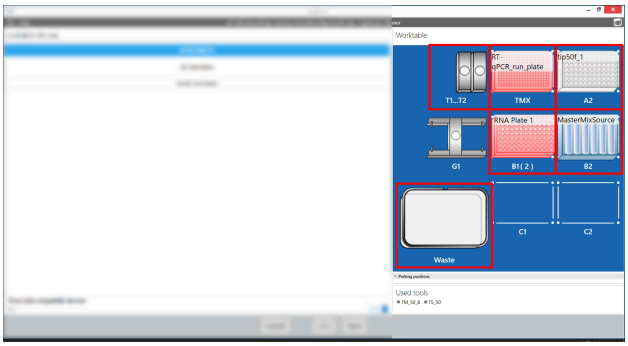
20 Input diluted Master Mix volumes. **Volumes must be accurate.** Click next to proceed.

Worktable Setup

21 Worktable positions used. Empty waste bin before use.

Note

Screenshot



22 Ensure that the TS-50 and TM-50 pipette heads are in positions T1 and T2. It does matter which spot each pipette head is in.

Note

Equipment Information:

Equipment

TS 50 single-channel dispensing tool	NAME
epMotion® Dispensing tools	TYPE
Eppendorf	BRAND
Catalog no. 960001010	SKU

Equipment

TM 50-8 eight-channel dispensing tool	NAME
epMotion® Dispensing tools	TYPE
Eppendorf	BRAND
Catalog No. 960001044	SKU

23 Insert a 384-well plate into the TMX slot. Press gently but ensure theplate is secure.
Eppendorf twin.tec® PCR Plates **Eppendorf Catalog #** 951020745

24 Place 96-well thermoadapter in slot B1.

Note

Equipment Information:

Equipment

Thermoadapter for PCR	NAME
epMotion Thermoracks and Plate Accessories	TYPE
Eppendorf	BRAND
Catalog No. 960002199	SKU

25 Place a 96-well PCR plate onto the thermoadapter already in position B1.
96 Well PCR Plates, Semi-Skirted **Phenix Research Catalog #**1147B52

26 Add tips to the worktable at position A2. These programs all use PCR clean p50 filter tips. The tip rack has marks on the side that indicate to the machine what tips are

present in the box. When placed onto the worktable, those marks need to be on the left.

Note

If the program requires more than 96 tips, the software will stop at that point and allow users to add more tips.

Note

Equipment Information:

Equipment	
epT.I.P.S.® Motion Pipette Tips	NAME
epMotion Pipette Tips	TYPE
Eppendorf	BRAND
Catalog No. 0030014413	SKU
P50	SPECIFICATIONS

27 Place ReservoirRack in position B2.

Note

Equipment Information:

Equipment	
ReservoirRack	NAME
epMotion® ReservoirRacks and Modules	TYPE
Eppendorf	BRAND
Catalog No. 960002148	SKU

28 Place the ReservoirRack Module PCR into the ReservoirRack. This module occupies six of the seven slots. The gap should be on the left side (position 1).

Note

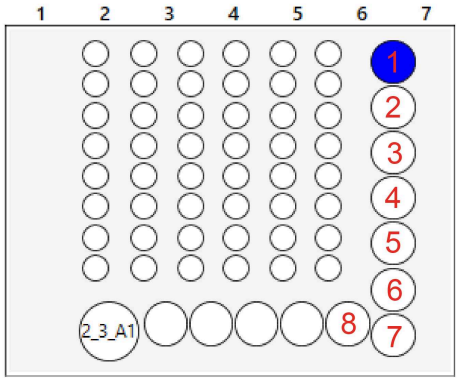
Equipment Information:

Equipment	
ReservoirRack Module PCR	NAME
epMotion® ReservoirRacks and Modules	TYPE
Eppendorf	BRAND
Catalog no. 5075751933	SKU

29 Place master mix tubes into designated slots (below) and input volumes into the software.
Input volume. The "Set all volumes" control does not work well for this step.

	Probe #	1 Target Program	2 Target Program	3 Target Program	4 Target Program	5 Target Program	6 Target Program	7 Target Program	8 Target Program
	1	2_2_A1	2_2_A1	2_2_A1	2_2_A1	2_2_A1	2_2_A1	2_2_A1	2_2_A1
	2		2_2_B1	2_2_B1	2_2_B1	2_2_B1	2_2_B1	2_2_B1	2_2_B1
	3			2_2_C1	2_2_C1	2_2_C1	2_2_C1	2_2_C1	2_2_C1
	4				2_2_D1	2_2_D1	2_2_D1	2_2_D1	2_2_D1
	5					2_2_E1	2_2_E1	2_2_E1	2_2_E1
	6						2_2_F1	2_2_F1	2_2_F1
	7							2_2_G1	2_2_G1
	8								2_4_A5

ReservoirRack Module PCR Master Mix Positions



Number indicates target order and position in the ReservoirRack Module PCR

Run

30 Click "Run." Users may wish to go and set up the RT-PCR machine with the experimental layout (see go to step #34)

Note

- During the run, users can pause and abort the run if they identify an issue.
- If the run is aborted, users will need to remove any pipette heads from the tool positions (next to TMX) so that the machine can return an attached pipette head.
- The software will analyze the run parameters and notify users of any potential issues (such as not enough tips). Should the tips run out during the run, the machine will stop and allow users to exchange tip boxes.

31 Click "OK" on the pop-up declaring "The application ended successfully".

32 Click "Exit."

33 Open the door, remove the 384-well plate, and seal.

RT-PCR

34 1000 rpm, 00:05:00 , 384-well plate, tabletop centrifuge

35 Set up RT-PCR machine plate layout.

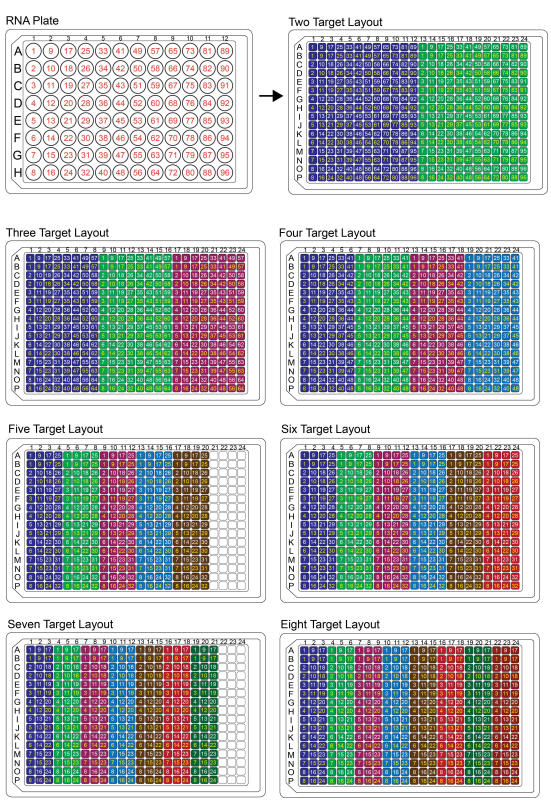


Figure 2. 384-well Plate Pattern.
Gene expression targets are ordered left to right and indicated by different-colored wells. White and yellow text indicate the technical duplicates for each sample and target.

36 Run the plate on QuantStudio™.

Note

Preparing the QuantStudio™ is not a part of this protocol.

Data Analysis

37 Export results as a CSV. Select all fields in the Results tab. This will make copying and pasting data in the next step easier.

38 Copy the results to an Excel template file. This template converts the exported long data format to a plate layout and duplicate format. Make sure the data is pasted into the template correctly.

ExpressionStudioConverter.xltx 43.1KB (newer software, uses Cq)

RT-PCR_DataConverter.xltx 41.7KB (for older software, uses Ct)

39 Exit to the Start Screen, go to the log viewer, and retrieve the PDF printout of the log file for the run.

Acknowledgements

We want to acknowledge Patrick Carney, Ksenija Korac, and Manabu Nukaya of the Sean Ronnekleiv-Kelly Lab for their assistance in validating our test protocols.