MAKING SCIENCE AFFORDABLE

PACE® ONESTEP RT-PCR MASTER MIX

USER GUIDE

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CONTENT

1. PRODUCT DETAILS

PRODUCT	PRODUCT VOLUME	PART NUMBER	REACTIONS AT 10 µL	CONCENTRATION
PACE® OneStep RT-PCR Master Mix (**Standard ROX** – 150 nM)	1 mL	RT-PACE-01	200	2x
	10 mL	RT-PACE-02	2,000	2x
	25 mL	RT-PACE-03	5,000	2x
	250 mL	RT-PACE-04	50,000	2x
PACE® OneStep RT-PCR Master Mix (**Low ROX** – 25 nM)	1 mL	RT-PACE-05	200	2x
	10 mL	RT-PACE-06	2,000	2x
	25 mL	RT-PACE-07	5,000	2x
	250 mL	RT-PACE-08	50,000	2x
PACE® OneStep RT-PCR Master Mix (**High ROX** – 500 nM)	1 mL	RT-PACE-09	200	2x
	10 mL	RT-PACE-10	2,000	2x
	25 mL	RT-PACE-11	5,000	2x
	250 mL	RT-PACE-12	50,000	2x
PACE® OneStep RT-PCR Master Mix (**no ROX**)	1 mL	RT-PACE-13	200	2x
	10 mL	RT-PACE-14	2,000	2x
	25 mL	RT-PACE-15	5,000	2x
	250 mL	RT-PACE-16	50,000	2x

2. DESCRIPTION

PACE OneStep RT-PCR Master Mix is designed for highly specific and sensitive one-step reverse transcriptase (RT) fluorescently-reporting endpoint PCR using RNA template. The master mix includes optimised components which allow reverse transcription and subsequent PCR amplification to take place in the same reaction well.

The PACE OneStep RT-PCR is comprised of three parts:

1. 72x PACE Genotyping Assay mix
2. Reverse transcriptase
3. Universal PACE OneStep RT-PCR Master Mix; containing all components required for PCR amplification (post-reverse transcription), and the generation of fluorescent signals.

When combined with template RNA, these components create a PACE OneStep RT-PCR.

3. STORAGE AND SHELF LIFE

PACE OneStep RT-PCR Master Mix is shipped on blue ice. Upon arrival, store at -20˚C/-80˚C (stable for two years); multiple freeze/thaw cycles are not recommended. The mix can also be stored at 4˚C for two weeks (protected from light). The Reverse Transcriptase (RT) should be stored at -20˚C and will retain full activity for 1 year.

4. SAFETY WARNINGS AND PRECAUTIONS

This product should only be handled by trained laboratory personnel. It is advisable to wear suitable personal protective equipment (PPE) when using the product. In case of contact with skin or eyes, wash immediately with water.

5. KIT COMPONENTS

- PACE OneStep RT-PCR Master Mix (supplied at 2x concentration) contains a specifically engineered Taq polymerase, universal fluorescent reporting cassette, dNTPs, buffer, performance enhancers, $MgCl_2$ at 4.4 mM (2.2 mM at 1x concentration) and passive reference dye (ROX).
- Reverse Transcriptase (RT) (200x)

REQUIRED COMPONENTS

- Fluorescent plate reader or qPCR machine capable of reading the fluorophores in *Table 1*
- PCR plate or equivalent and appropriate optically clear seal
- Template RNA
- PCR-grade water
- PACE Genotyping Assays (see section 8).

FLUOROPHORE	EXCITATION (NM)	EMISSION (NM)
FAM	485	520
HEX	520	560
ROX *	580	610

Table 1. Excitation and Emission values for the fluorophores used in the PACE OneStep RT-PCR Master Mix.

** Only required where appropriate; see www.3crbio.com/faqs*

6. ROX COMPATIBILITY

PACE OneStep RT-PCR Master Mix is supplied without ROX, or with standard, low or high ROX levels. Please ensure compatibility between the ROX level of the master mix and the qPCR instrument. Should you require further assistance, please contact your qPCR instrument/plate reader manufacturer, or contact 3CR Bioscience's Technical Support team. If a fluorescent plate reader is used instead of a qPCR machine, it is recommended that the standard ROX version of the PACE OneStep RT-PCR Master Mix is used.

7. MECHANISM OF ACTION

The assays used with PACE OneStep RT-PCR Master Mix are comprised of a forward primer(s) incorporating an un-labeled 5' tail sequence and a reverse primer(s). During the reverse transcription phase, the assay primers facilitate targeted cDNA synthesis from the template RNA.

PACE OneStep RT-PCR Master Mix uses a universal, quenched fluorescent reporting cassette to produce machine-readable fluorescent signals. Once the PCR phase is initiated, the reaction proceeds as for the standard PACE chemistry, using the newly synthesised cDNA as the template. As PCR proceeds, the tail sequences of the forward primers become incorporated into the amplicon and the corresponding tail sequence complements are generated. At this point the reporting cassettes bind to their tail sequence complements, becoming unquenched and producing a light signal (see the PACE Genotyping Master Mix User Guide Section 7 for more details).

8. ASSAYS

ASSAY COMPONENT	VOLUME TO ADD FOR 100 µL	FINAL CONC. (µM)
PACE Genotyping Assay 1 Forward primer (FAM tail-sequence) (100 µM)	12	12
PACE Genotyping Assay 1 Reverse primer (100 µM)	30	30
PACE Genotyping Assay 2 Forward primer (HEX tail-sequence) (100 µM)	12	12
PACE Genotyping Assay 2 Reverse primer (100 µM)	30	30
Nuclease-free water	16	-
TOTAL	**100**	**-**

Table 2. **Reagent volumes for assembly of PACE Genotyping Assay mix. A mix of two assays is shown; if only one assay is required the water volume can be increased accordingly but the final assay concentration should remain the same.**
Note that *Table 2* describes assays for presence/absence sequence detection. For details of assay set up for SNP/Indel genotyping applications, please refer to the PACE Genotyping Master Mix User Guide (section 8).

A complimentary PACE Genotyping Assay design service is available at www.3crbio.com. KASP™ and Amplifluor® assays can also be used in conjunction with the PACE OneStep RT-PCR chemistry, as the 5' tail sequences are the same. *Table 2* shows the assembly of a PACE OneStep RT-PCR assay mix from the constituent primers.

9. RNA QUALITY AND QUANTITY

RNA is highly susceptible to ubiquitous RNases. Care should be taken when handling the samples. An empirical optimisation of RNA concentration should be carried out by testing a sample dilution range.

If RNA samples contain EDTA, the concentration at the final reaction concentration should be no higher than 0.1 mM.

10. CONTROLS

To improve confidence in the genotyping data, control samples should be used on the PCR plate in addition to the test samples. Negative controls (no-template controls, or NTCs) should always be used and consist of the same buffer used to hydrate the RNA samples, dispensed into several wells of the PCR plate. Positive controls can also be used, if available, and should consist of RNA samples of known sequence.

When viewing the data, NTCs should show no amplification, giving confidence that any amplification observed is real. Any amplification observed in the NTC wells would indicate contamination or non-specific amplification. The positive control samples should amplify as expected for their sequence.

11. REACTION PROCEDURE

A. ARRAYING TEMPLATE RNA

A liquid-handling system appropriate to the number of RNA samples to array should be used. RNA samples can also be arrayed manually if working with a small number of samples.

B. PACE ONESTEP RT-PCR ASSEMBLY

Ensure the components are defrosted thoroughly, mix and centrifuge briefly prior to use. The enzymes used in PACE OneStep RT-PCR Master Mix are modified such that they are completely inactive at ambient temperature. Such inactivation allows bench top reaction assembly without leading to primer-dimer issues and other side reactions. The enzymes are reactivated during the initial stage of thermal cycling.

PACE OneStep RT-PCR Master Mix can be used with any reaction plate or well volume. It is very important that PACE OneStep RT-PCR Master Mix is used at a final 1x concentration. However, the exception to this is in the PACE Genotyping Assay mix volumes shown in *Table 3* - the PACE Genotyping Assay mix volume is ignored as it does not cause any functionally meaningful dilution and doesn't affect the reaction performance.

COMPONENT	CONSTITUTION OF 5 µL REACTION (µL)	VOLUME FOR EXEMPLAR 250 µL TOTAL REACTION MIX (µL)
PACE OneStep RT-PCR Master Mix (2x)	2.5	125
Reverse transcriptase (RT) (200x)	0.025	1.25
PACE Genotyping Assay mix (72x)	0.07	3.47
RNA	1	-
Nuclease-free water	1.41	70.28
TOTAL	**5**	**200 µL** *(250 µL including RNA volume)*

Table 3. Assembly of a PACE OneStep RT-PCR. As indicated, a PACE OneStep RT-PCR mix should be made to avoid pipetting error. In the example given, a 200 µL total reaction mix is made and 4.0 µL is added to the 384-well plate wells, followed by 1.0 µL of RNA sample to each well. Adjust volumes appropriately if using a 96-well plate.

C. TOTAL PACE ONESTEP RT-PCR MIX DISPENSING & PLATE SEALING

The total reaction mix (*Table 3*) must now be dispensed into the PCR-plate wells using a liquid-handling system appropriate to the scale of the work. Once the total reaction mix has been dispensed, the PCR plate must be sealed with an optically clear seal and centrifuged to ensure all components are at the bottom of the wells.

PACE OneStep RT-PCR Master Mix can be used with any standard qPCR instrument, Peltier or water bath-based thermal cycler. The fluorescent data should be captured at the PCR endpoint at 40˚C or below. Use the thermal-cycling protocol detailed in *Table 4a*.

D. THERMAL CYCLING

The PACE OneStep RT-PCRs must be thermally cycled according to the protocol described in *Table 4a*.

STEP	DESCRIPTION	TEMP.	TIME	NO. CYCLES
1	Reverse transcription	50˚C	10-30min	1
2	Enzyme activation	94˚C	15 min	1
3	Template denaturation	94˚C	20 secs	10
	Annealing and extension	65-57˚C	60 secs (drop 0.8˚C per cycle)	
4	Denaturation	94˚C	20 secs	30
	Annealing and extension	57˚C	60 secs	

Table 4a. Thermal cycling conditions for PACE OneStep RT-PCR Master Mix

DESCRIPTION	TEMPERATURE	TIME	CYCLES PER STEP
Template denaturing	94˚C	20 secs	3
Annealing and extension	57˚C	60 secs	

Table 4b. Thermal cycling conditions for recycling PACE OneStep RT-PCRs

E. DATA COLLECTION

The fluorescent signal data should be collected using an appropriate fluorescent plate reader or qPCR instrument. The data can be analysed in a cluster analysis software package.

If the data clusters are not sufficiently defined after running the initial thermal-cycling protocol, the plate should be cycled for an additional three cycles (see *Table 4b*) then the fluorescent signal data collected again. The additional cycling/data analysis can be repeated until tight and well-separated clusters are observed, however it is recommended that this step is repeated a maximum of four times.

It is important that the fluorescent signal is read at or below 40˚C. If using a qPCR instrument, an additional temperature-controlled reading step should be included after the final PCR step or used separately to it. The temperature-controlled reading step should be used with both the main PCR (as described in *Table 4a*) and with any subsequent additional cycling steps (*Table 4b*). If using a fluorescent plate reader, the addition of this temperature-controlled reading step to the thermal-cycling protocol should not be necessary as the PCR plate will have cooled sufficiently by the plate-reading stage.

F. INTERPRETATION OF DATA

The HEX and FAM florescence signal data produced by PACE OneStep RT-PCR should be analysed and interpreted as a cluster plot using cluster analysis software or with Microsoft Excel (see *Figure 1*).

ROX passive reference dye can also be used to eliminate the effect of well-to-well liquid volume differences from the resulting cluster plot data. The inclusion of a passive reference leads to tighter clustering and, as a result, more accurate scoring of data.

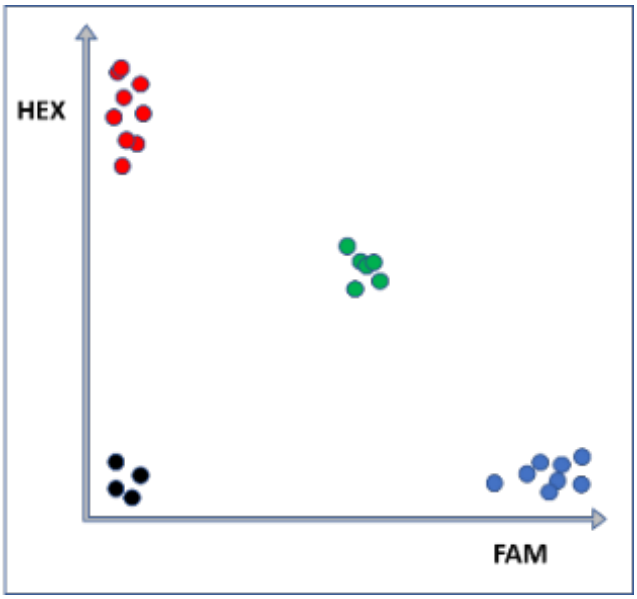

Figure 1. Diagram of typical genotyping cluster plot data generated from a PACE OneStep RT-PCR. Black samples at the origin are the no-template controls (NTCs).

12. ORDERING INFORMATION

For ordering details, please visit www.3crbio.com

13. SUPPORT

If you require any support with the use of PACE OneStep RT-PCR Master Mix or other 3CR Bioscience products, please contact our Technical Support team on support@3crbio.com.

14. LEGAL INFORMATION

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