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PACE® MULTIPLEX MASTER MIX

USER GUIDE

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CONTENT

1. PRODUCT DETAILS

PRODUCT	PRODUCT VOLUME	PART NUMBER	NUMBER OF REACTIONS AT 10 µL	CONCENTRATION
PACE® Multiplex Master Mix (**Standard ROX** – 150 nM)	2.5 mL	007-0001	500	2x
	25 mL	007-0002	5,000	2x
	250 mL	007-0003	50,000	2x
	1,000 mL	007-0004	200,000	2x
PACE® Multiplex Master Mix (**Low ROX** – 25 nM)	2.5 mL	007-0005	500	2x
	25 mL	007-0006	5,000	2x
	250 mL	007-0007	50,000	2x
	1,000 mL	007-0008	200,000	2x
PACE® Multiplex Master Mix (**High ROX** – 500 nM)	2.5 mL	007-0009	500	2x
	25 mL	007-0010	5,000	2x
	250 mL	007-0011	50,000	2x
	1,000 mL	007-0012	200,000	2x
PACE® Multiplex Master Mix (**No ROX**)	2.5 mL	007-0013	500	2x
	25 mL	007-0014	5,000	2x
	250 mL	007-0015	50,000	2x
	1,000 mL	007-0016	200,000	2x
PACE® Multiplex Master Mix (**1x concentration Standard ROX** – 150 nM)	250 mL	007-0017	25,000	**1x**

2. DESCRIPTION

PACE Multiplex (**P**CR **A**llelic **C**ompetitive **E**xtension) genotyping chemistry is a homogeneous, PCR-based allele-specific technology for the simultaneous analysis of two Single Nucleotide Polymorphisms (SNPs) or insertion/deletions (Indels), or detection of sequences of interest.

The PACE Multiplex chemistry is comprised of two parts:

1. PACE Genotyping Assay: comprising two pairs of allele-specific forward primers and two common, reverse primers for SNPs or Indels, or four pairs of primers for detecting genes of interest.
2. PACE Multiplex Master Mix: containing all components required for PCR and generation of fluorescent signals.

When combined and mixed with template DNA, these components create a PACE Multiplex Genotyping Reaction.

3. STORAGE AND SHELF LIFE

PACE Multiplex 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. PACE Multiplex Master Mix can be aliquoted into light-protective tubes to reduce the need for repeated freeze-thaw cycles. The mix can also be stored at 4˚C for two weeks (protected from light).

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 Multiplex Master Mix (supplied at 2x concentration), containing a specifically engineered Taq polymerase, universal fluorescent reporting cassettes, dNTPs, buffer, performance enhancers, $MgCl_2$ at 4.4 mM (2.2 mM at 1x concentration) and the passive reference dye, ROX.

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 DNA
- PCR-grade water
- Genotyping assays (see section 8).

FLUOROPHORE	EXCITATION (nM)	EMISSION (nM)
FAM	485	520
HEX	520	560
ATTO 550	560	575
ATTO 647N	649	662
ROX *	580	610

Table 1. Excitation and Emission values for the fluorophores used in the PACE Multiplex chemistry.

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

6. ROX COMPATIBILITY

PACE Multiplex 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 the manufacturer of your qPCR instrument or plate reader or contact 3CR Bioscience's Technical Support team.

If a fluorescent plate reader is used instead of a qPCR instrument, it is recommended that the standard ROX version of PACE Multiplex Master Mix is used.

7. MECHANISM OF ACTION

PACE Multiplex Master Mix uses a novel, universal, fluorescent reporting cassette to produce machine-readable fluorescent signal. The master mix contains four universal quenched fluorescent reporting cassettes, labelled with the fluorophores FAM, HEX, ATTO 550 and ATTO 647N.

When used for genotyping applications, two genotyping assays are used, each comprised of two competitive allele-specific forward primers (which differ in their terminal 3' bases and unique 5' tail sequences), and a common reverse primer. One multiplex assay reports with FAM and HEX, whilst the other reports with ATTO 550 and ATTO 647N.

When PCR is initiated, the allele-specific primers bind with their 3' ends at the SNP of interest. Both allele-specific primers will bind if the SNP is heterozygous, whereas only one or other of the primers will bind if the SNP is homozygous. At the same time, the common reverse primer will bind on the opposite strand.

As PCR proceeds, the tail sequences of allele-specific forward primers become incorporated into the amplicon and the corresponding tail sequence complement is generated. At this point the quenched, fluorescent reporting cassettes bind to their appropriate tail sequence complements, becoming unquenched and producing a light signal. If the genotype of the SNP is homozygous, only one of the possible fluorescent signals will be generated, whereas if the SNP is heterozygous, the result will be a mixed fluorescent signal.

Alternatively, if PACE Multiplex Master Mix is used for sequence detection applications, one forward primer (containing a 5' tail sequence, as for the allele-specific primers in the genotyping application above) can be designed to a reference sequence whilst the other three forward primers can be designed to individual targets of interest, with a reverse primer designed for each PCR. When testing for the presence or absence of sequences, the forward and reverse primers will bind, and the PCR will proceed as described above for a SNP.

The reference gene will always be present, so the assay will produce a homozygous fluorescent signal for the fluorophore selected for the reference sequence assay; this shows that the reaction is functioning as expected. The other assay primers will bind to their target sequences of interest (if present) and the appropriate signal(s) will be produced. If a gene of interest is present, both signals will be generated, resulting in a heterozygous sample.

8. MULTIPLEX ASSAYS

PACE Genotyping Assay designs are available free-of-charge by using our complimentary PACE Assay design service at www.3crbio.com or by contacting support@3crbio.com

A. GENOTYPING ASSAYS

PACE Multiplex Master Mix allows the use of two PACE Genotyping Assays simultaneously in one reaction well. *Tables 2a and 2b* show how to assemble assay mixes for PACE Genotyping Assays 1 and 2 from the constituent primers.

Pre-existing KASP™ and Amplifluor® assays (i.e., which contain allele-specific primers with FAM/HEX reporting tails) can also be used in conjunction with the PACE Multiplex genotyping chemistry. If used, these assays would replace those described in *Table 2a*.

PACE GENOTYPING ASSAY 1		
PRIMER	FINAL CONCENTRATION (µM)	VOL. REQUIRED FOR 100 µL ASSAY MIX (µL)
Assay 1 allele-specific forward primer 1 – FAM sequence tail (100 µM)	12	12
Assay 1 allele-specific forward primer 2 – HEX sequence tail (100 µM)	12	12
Assay 1 common, reverse primer (100 µM)	30	30
PCR-grade water	-	46
TOTAL	**-**	**100**

Table 2a. Assembly of PACE Multiplex Genotyping Assay 1 (reporting with FAM and HEX).

PACE GENOTYPING ASSAY 2		
PRIMER	FINAL CONCENTRATION (µM)	VOL. REQUIRED FOR 100 µL ASSAY MIX (µL)
Assay 2 allele-specific forward primer 1 – ATTO 550 sequence tail (100 µM)	12	12
Assay 2 allele-specific forward primer 2 – ATTO 647N sequence tail (100 µM)	12	12
Assay 2 common, reverse primer (100 µM)	30	30
PCR-grade water	-	46
TOTAL	**-**	**100**

Table 2b. Assembly of PACE Multiplex Genotyping Assay (reporting with ATTO 550 and ATTO 647N).

B. SEQUENCE DETECTION ASSAYS

Table 3 shows how to assemble a PACE Genotyping Assay mix to detect multiple targets of interest.

ASSAY	PRIMERS	FINAL CONCENTRATION (µM)	VOL. REQUIRED FOR 100 µL ASSAY MIX (µL)
Reference sequence	Forward primer **HEX** sequence tail (200 µM)	12	6
	Reverse primer (200 µM)	30	15
Target of interest 1	Forward primer **FAM** sequence tail (200 µM)	12	6
	Reverse primer (200 µM)	30	15
Target of interest 2	Forward primer **ATTO 550** sequence tail (200 µM)	12	6
	Reverse primer (200 µM)	30	15
Target of interest 3	Forward primer **ATTO 647N** sequence tail (200 µM)	12	6
	Reverse primer (200 µM)	30	15
	PCR-grade water	-	16
	Total	**-**	**100**

Table 3. **Assembly of PACE Genotyping Assay mix for detection of a reference sequence and three sequences of interest. Note that the primer stocks are at 200 µM to allow requisite final primer concentrations.**

9. DNA QUALITY AND QUANTITY

It is recommended to use 1-10 ng of gDNA per reaction well, though this will vary with organism genome size (large genomes will require a proportionately larger DNA mass). For optimal results, purified and well-normalised DNA samples should be used. However, when using PACE Multiplex Master Mix in high throughput, purified DNA is often not commercially practical. DNA that has been crudely extracted works well with PACE Multiplex Master Mix, but such samples should be tested before commencing large-scale work. Empirical optimisation of DNA concentration by testing a sample dilution range test is the most sensible approach. If DNA 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 DNA samples, dispensed into several wells of the PCR plate. Positive controls can also be used, if available, and should consist of DNA samples of known genotype.

When viewing the genotyping data, NTCs should show no amplification and remain around the origin of the cluster plot (see *Figure 1*), 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 cluster in the expected regions for their genotype.

11. REACTION PROCEDURE

A. ARRAYING TEMPLATE DNA

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

PACE Multiplex Master Mix can be used with hydrated (*Table 3a*) or dry (*Table 3b*) DNA samples. Both approaches work equally well but have practical advantages and disadvantages. If low numbers of samples are to be genotyped, it is not worth drying the DNA samples.

However, if high numbers of samples are to be genotyped in one run, drying the DNA samples can improve the resulting genotyping data. Hydrated DNA arrayed in a PCR plate will quickly begin to evaporate differentially across the plate (samples near the edges evaporate more quickly than those in the middle). Variation in DNA volumes across the plate will lead to variation in the final reaction concentrations, causing sub-optimal genotyping results. For this reason, when drying the DNA into the plate wells the user must ensure that the DNA has been dried to completion. Dried DNA samples will be stable long term at ambient temperature.

If a very small reaction volume is to be used (for example 1.0 µL total volume), it might not be possible to accurately dispense 0.5 µL of DNA and 0.5 µL of total reaction mix. In this example, drying the sample would allow a more realistic 1.0 µL of total reaction mix to be dispensed to the well.

To dry the DNA, once dispensed into a PCR plate, the plate should be centrifuged to ensure the samples are in the bottom of the wells and placed in a laboratory fan oven for one hour at around 55°C, or until the samples have visibly dried. When assembling the total reaction mix, water must be added in the correct proportion to account for the missing volume of the DNA template. See *Tables 3a and 3b* for details.

B. REACTION ASSEMBLY

PACE Multiplex Master Mix can be used with any reaction plate or well volume; it is not necessary to use a different product depending on reaction volume.

It is very important that PACE Multiplex Master Mix is used at a final 1x concentration. However, the exception to this is in the PACE Genotyping Assay volumes shown in *Tables 4a & 4b* - the PACE Genotyping Assay volume is ignored as it does not cause any functionally meaningful dilution and does not affect the performance.

	HYDRATED DNA METHOD (µL PER WELL)		
	96-WELL PLATE	384-WELL PLATE	384-WELL ARRAY TAPE
2X PACE MULTIPLEX MM	5.0	2.5	0.8
PACE ASSAY 1	0.138	0.069	0.022
PACE ASSAY 2	0.138	0.069	0.022
WATER	N/A	N/A	N/A
DNA	5.0	2.5	0.8
TOTAL	**10.0**	**5.0**	**1.6**

Table 4a. Reagent assembly for PACE Multiplex Genotyping Reaction with hydrated DNA.

	DRIED DNA METHOD (µL PER WELL)			
	96-WELL PLATE	384-WELL PLATE	1536-WELL PLATE	384-WELL ARRAY TAPE
2X PACE MULTIPLEX MM	5.0	2.5	0.5	0.4
PACE ASSAY 1	0.138	0.069	0.014	0.011
PACE ASSAY 2	0.138	0.069	0.014	0.011
WATER	5	2.5	0.5	0.4
DNA	N/A	N/A	N/A	N/A
TOTAL	**10.0**	**5.0**	**1.0**	**0.8**

Table 4b. Reagent volumes for PACE Multiplex Genotyping Reaction with dried DNA.
Note: 1536-well plates cannot be used with hydrated DNA because of sample evaporation issues, so are only used with the dried DNA method.

If using PACE Multiplex Master Mix for sequence detection applications, just one PACE Genotyping Assay mix would be used so the 'PACE Assay 2' in *Tables 4a & 4b* would be ignored.

C. PACE MULTIPLEX GENOTYPING REACTION DISPENSING & PLATE SEALING

The total reaction mix must now be dispensed into the PCR plate wells. As with DNA dispensing, use a liquid handling system that is 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.

D. THERMAL CYCLING

PACE Multiplex Genotyping Reactions should be thermally cycled according to the protocol described in *Table 5a*.

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

Table 5a. Thermal cycling conditions for PACE Multiplex Genotyping Reactions.

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

Table 5b. Thermal cycling conditions for recycling PACE Multiplex Genotyping Reactions.

E. FLUORESCENT SIGNAL DETECTION

After thermal cycling is complete, the fluorescent signal data should be collected using an appropriate fluorescent plate reader or qPCR machine in endpoint mode.

Table 6 details the optimal suggested wavelength and bandwidth settings for each filter to minimise crosstalk between the different filters.

FLUOROPHORE	EXCITATION (nM)	EMISSION (nM)
FAM	485 (7.5 nM)	520 (10 nM)
HEX	525 (7.5 nM)	550 (10 nM)
ATTO 550	560 (10 nM)	590 (10 nM)
ATTO 647	635 (10 nM)	670 (10 nM)
ROX	580 (10 nM)	610 (10 nM)

Table 6. Suggested excitation and emission filter settings (with bandwidth in brackets) for the fluorophores used in the PACE Multiplex chemistry.

If the genotype clusters are not sufficiently defined after running the initial thermal cycling protocol, the plate should be cycled for an additional three cycles (see *Table 5b*) 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 5a*) and with any subsequent additional cycling steps (*Table 5b*). 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, FAM, ATTO 550, and ATTO 647N florescence signal data produced by PACE Multiplex Genotyping Reactions 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.

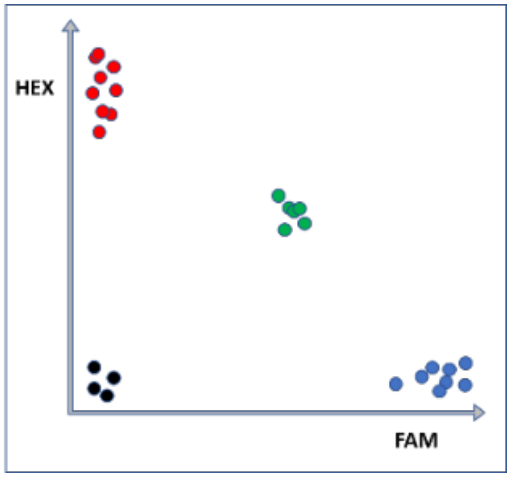

Figure 1. Diagram of typical genotyping cluster plot data generated from a PACE Multiplex Genotyping Reaction (only FAM / HEX plot shown). Black samples at the origin are the no-template controls (NTCs).

12. ORDERING INFORMATION

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

13. SUPPORT

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

14. LEGAL INFORMATION

For Research Use Only. Not for use in diagnostic procedures.

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