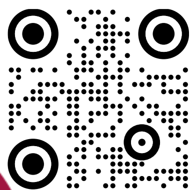

MAKING SCIENCE
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PACE® 2.0 GENOTYPING MASTER MIX

USER GUIDE

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CONTENT

1. PRODUCT DETAILS 3
2. DESCRIPTION 3
3. ADDITIONAL BENEFITS OF PACE 2.0 GENOTYPING MASTER MIX 4
4. STORAGE AND SHELF LIFE 4
5. SAFETY WARNINGS AND PRECAUTIONS 4
6. KIT COMPONENTS 4
7. ROX COMPATIBILITY 5
8. MECHANISM OF ACTION 5
9. GENOTYPING ASSAYS 5
10. DNA QUALITY AND QUANTITY 6
11. CONTROLS 6
12. GENOTYPING PROCEDURE 6
a. Arraying template DNA 6
b. Reaction assembly 7
c. Total reaction mix dispensing & plate sealing 8
d. Thermal cycling 8
e. Fluorescent signal detection 9
f. Interpretation of data 9
13. ORDERING INFORMATION 9
14. SUPPORT 10
15. LICENCE INFORMATION 10

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1. PRODUCT DETAILS

PRODUCT	PRODUCT VOLUME	PART NUMBER	NUMBER OF REACTIONS AT 10 µL	CONCENTRATION
PACE® 2.0 Genotyping Master Mix (**Standard ROX** – 150 nM)	2.5 mL	003-0001	500	2x
	25 mL	003-0002	5,000	2x
	250 mL	003-0003	50,000	2x
	1,000 mL	003-0004	200,000	2x
PACE® 2.0 Genotyping Master Mix (**Low ROX** – 25 nM)	2.5 mL	003-0005	500	2x
	25 mL	003-0006	5,000	2x
	250 mL	003-0007	50,000	2x
	1,000 mL	003-0008	200,000	2x
PACE® 2.0 Genotyping Master Mix (**High ROX** – 500 nM)	2.5 mL	003-0009	500	2x
	25 mL	003-0010	5,000	2x
	250 mL	003-0011	50,000	2x
	1,000 mL	003-0012	200,000	2x
PACE® 2.0 Genotyping Master Mix (**no ROX**)	2.5 mL	003-0013	500	2x
	25 mL	003-0014	5,000	2x
	250 mL	003-0015	50,000	2x
	1,000 mL	003-0016	200,000	2x
PACE® 2.0 Genotyping Master Mix (1x concentration, **Standard ROX** – 150 nM)	250 mL	003-0017	25,000	**1x**
	1,000 mL	003-0018	100,000	**1x**

2. DESCRIPTION

PACE 2.0 (**P**CR **A**llelic **C**ompetitive **E**xtension) genotyping chemistry is a homogeneous, PCR-based allele-specific technology for the analysis of Single Nucleotide Polymorphisms (SNPs) and insertion / deletions (Indels).

The PACE 2.0 genotyping chemistry is comprised of two parts:

1. SNP-specific assay mix; comprising two allele-specific forward primers and one common, reverse primer.
2. PACE 2.0 Genotyping Master Mix; containing all components required for PCR and generation of fluorescent signals.

When combined with template DNA, these components create a PACE 2.0 genotyping reaction.

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3. ADDITIONAL BENEFITS OF PACE 2.0 GENOTYPING MASTER MIX

- Higher signal-to-noise, giving higher fluorescent values, enabling the user to see improved group separation during analysis.
- Inhibitor resistance enables this mix to be used with crudely extracted DNA samples as well as purified DNA samples.

4. STORAGE AND SHELF LIFE

PACE 2.0 Genotyping 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 2.0 Genotyping 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).

5. 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.

6. KIT COMPONENTS

PACE 2.0 Genotyping Master Mix (supplied at 2x concentration), containing 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 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 9).

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 2.0 genotyping chemistry.

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

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7. ROX COMPATIBILITY

PACE 2.0 Genotyping 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 the PACE 2.0 Genotyping Master Mix is used.

8. MECHANISM OF ACTION

PACE 2.0 uses a novel, universal, fluorescent reporting cassette to produce machine-readable fluorescent signals corresponding to genotypes. A genotyping assay is 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. The PACE 2.0 Genotyping Master Mix contains a quenched fluorescent reporting cassette for the fluorophores FAM and HEX.

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.

9. GENOTYPING ASSAYS

Genotyping assay designs are available by using our free assay design service at www.3crbio.com.

PACE 2.0 uses the same assays as our standard PACE product. Pre-existing KASP™ and Amplifluor® assays can also be used in conjunction with the PACE 2.0 genotyping chemistry. *Table 2* shows how to assemble a genotyping assay mix from the constituent primers.

PRIMER	FINAL CONCENTRATION (µM)	VOL REQUIRED FOR 100 µL ASSAY MIX (µL)
Allele-specific primer 1 – FAM sequence tail (100 µM)	12	12
Allele-specific primer 2 – HEX sequence tail (100 µM)	12	12
Common, reverse primer (100 µM)	30	30
PCR-grade water	-	46
Total	**-**	**100**

Table 2. Assembly of a genotyping assay mix.

10. 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 2.0 in high throughput, purified DNA is often not commercially practical. DNA that has been crudely extracted works particularly well with PACE 2.0, 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.

11. 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 genotypes.

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.

12. GENOTYPING 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 low number of samples.

PACE 2.0 Genotyping Master Mix can be used with hydrated (*Table 3*) or dry (*Table 4*) 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 into 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 then 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 *Table 4* for details.

B. REACTION ASSEMBLY

PACE 2.0 Genotyping 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 2.0 Genotyping Master Mix is used at a final 1x concentration. However, the exception to this is in the assay mix volumes shown in *Tables 3 & 4*, where the assay mix volume is ignored as it does not cause any functionally meaningful dilution and doesn't affect the performance.

	HYDRATED DNA METHOD (µL PER WELL)		
	96-WELL PLATE	384-WELL PLATE	384-WELL ARRAY TAPE
2X PACE 2.0 GMM	5.0	2.5	0.8
ASSAY MIX	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 3. Reagent volumes for total reaction mix with hydrated DNA.

	DRIED DNA METHOD (µL PER WELL)			
	96-WELL PLATE	384-WELL PLATE	1536-WELL PLATE	384-WELL ARRAY TAPE
2X PACE 2.0 GMM	5.0	2.5	0.5	0.4
ASSAY MIX	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 4. Reagent volumes for total reaction mix 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.

C. TOTAL REACTION MIX 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

The PACE 2.0 genotyping reactions must be thermally cycled according to the protocol described in *Table 5.*

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 5. Thermal cycling conditions for PACE 2.0 genotyping reactions.

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

Table 6. Thermal cycling conditions for recycling PACE 2.0 genotyping reactions.

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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.

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 6*) 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 5*) and with any subsequent additional cycling steps (*Table 6*). 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 2.0 Genotyping Master Mix 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.

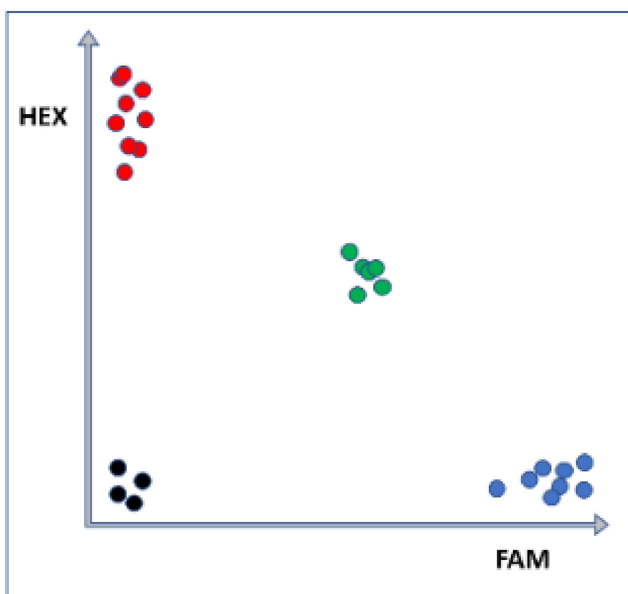

Figure 1. Diagram of typical genotyping cluster plot data generated from a PACE 2.0 genotyping reaction. Black samples at the origin are the no-template controls (NTCs).

13. ORDERING INFORMATION

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

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14. SUPPORT

If you require any support with the use of PACE 2.0 Genotyping Master Mix or other 3CR Bioscience products, please contact our Technical Support team on support@3crbio.com.

15. LICENCE INFORMATION

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