PACE™ Genotyping Master Mix User Guide

Content

1. Product details

Product	Product volume	Part number	Reactions	Reaction volume*	Concentration
PACE™ Genotyping Master Mix (**Standard ROX** – 150nM)	2.5 mL	001-0001	500	10 µL	2x
	25 mL	001-0002	5,000	10 µL	2x
	250 mL	001-0003	50,000	10 µL	2x
	1,000 mL	001-0004	200,000	10 µL	2x
PACE™ Genotyping Master Mix (**Low ROX** – 25nM)	2.5 mL	001-0005	500	10 µL	2x
	25 mL	001-0006	5,000	10 µL	2x
	250 mL	001-0007	50,000	10 µL	2x
	1,000 mL	001-0008	200,000	10 µL	2x
PACE™ Genotyping Master Mix (**High ROX** – 500nM)	2.5 mL	001-0009	500	10 µL	2x
	25 mL	001-0010	5,000	10 µL	2x
	250 mL	001-0011	50,000	10 µL	2x
	1,000 mL	001-0012	200,000	10 µL	2x
PACE™ Genotyping Master Mix (**no ROX**)	2.5 mL	001-0013	500	10 µL	2x
	25 mL	001-0014	5,000	10 µL	2x
	250 mL	001-0015	50,000	10 µL	2x
	1,000 mL	001-0016	200,000	10 µL	2x
PACE™ Genotyping Master Mix (**Standard ROX** – 150nM)	250 mL	001-0017	25,000	10 µL	**1x**
	1,000 mL	001-0018	100,000	10 µL	**1x**

* PACE™ Genotyping Master Mix has been used and tested successfully in volumes as low as 0.8 µL

2. Description

PACE™ (PCR Allelic Competitive Extension) genotyping chemistry is a homogeneous, PCR-based allele-specific technology for the analysis of Single Nucleotide Polymorphisms (SNPs) and insertion / deletions (indels).

The PACE™ genotyping chemistry is comprised of two parts:

1. SNP-specific assay mix; comprising two allele-specific forward primers and one common, reverse primer.
2. Universal PACE™ Genotyping 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™ genotyping reaction.

3. Storage and shelf life

3CR Bioscience's PACE™ Genotyping Master Mix is shipped on blue ice. Upon arrival, store at -20˚C (stable for one year); multiple freeze / thaw cycles are not recommended. The mix can also be stored at 4˚C for four 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 Genotyping Master Mix (supplied at 2x concentration), containing Taq polymerase, universal fluorescent reporting cassette, dNTPs, buffer, performance enhancers, $MgCl_2$ at 4.4 mM (2.2mM at 1x concentration) and the passive reference dye, 5-carboxy-X-rhodamine, succinimidyl ester (ROX).

Not included in the kit

- 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
ROX*	580	610

Table 1. **Excitation and Emission values for the fluorophores used in the PACE™ genotyping chemistry.**
*** Only required where appropriate; see www.3crbio.com/faqs**

6. ROX compatibility

PACE™ Genotyping Master Mix is supplied at standard, low or high ROX levels. Please ensure compatibility between the ROX level of the master mix and the qPCR machine – should you require further assistance, please contact your reader manufacturer or 3CR Bioscience.

If a fluorescent plate reader is to be used instead of a qPCR machine, it is recommended that the standard ROX version of the PACE™ Genotyping Master Mix is used.

7. Mechanism of action

The PACE™ Genotyping Master Mix chemistry uses a novel, universal, fluorescent reporting cassette to produce machine-readable fluorescent signals corresponding to genotypes. A PACE™ 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™ Genotyping Master Mix contains a quenched fluorescent reporting cassette for the fluorophores FAM and HEX. When PCR is initiated, the allele-specific primers bind upstream of the target SNP, with their 3' ends at the site of the SNP. Both 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 downstream of the SNP, on the opposite strand. As PCR proceeds, the tail sequences of allele-specific forward primers that have bound become incorporated upstream of the SNP and the corresponding tail sequence complement is generated. At this point reporting cassettes bind to the appropriate upstream tail sequence complement, 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.

8. Genotyping assays

Genotyping assay designs are available by using our free assay design service at www.3crbio.com/free-assay-design.
KASP™, Amplifluor®, etc… assays can also be used in conjunction with the PACE™ genotyping chemistry. *Table 2* shows how to assemble a PACE genotyping assay mix from the constituent primers.

Primer	Final concentration (µM)	Vol required for 100 µL assay mix (µL)
Allele-specific primer 1 – FAM (100 µM)	12	12
Allele-specific primer 2 – HEX (100 µM)	12	12
Common, reverse primer (100 µM)	30	30
PCR grade water	-	46
Total	**-**	**100**

***Table 2*. Assembly of PACE™ genotyping assay mix.**

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™ Genotyping Master Mix in high throughput, purified DNA is often not commercially practical. DNA that has been crudely extracted by methods such as hotSHOT works well with PACE™ Genotyping Master Mix, but these should be tested before commencing large scale work. Empirical optimization of DNA concentration by testing a sample dilution range test is the most sensible approach. Such testing is particularly important when using crude lysates as these may contain inhibitory substances at concentrations that are too high for the mix formulation to tolerate. In this case the optimal concentration will be where the level of any sample-derived inhibitors is sufficiently reduced to allow PCR to proceed. In some rare cases, the DNA concentration remaining at this dilution point can be too low for PCR to proceed, in which case the samples should be purified. If DNA samples contain EDTA, the concentration at the final reaction concentration should be no higher than 0.1 mM.

10. Controls

To have 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 (NTCs)) should always be used and consist simply 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 be DNA samples of known genotype.

When viewing the genotyping data, NTCs should show no amplification and remain at the origin of the cluster plot (see *Figure 1*), giving confidence that any amplification observed is true. 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. Genotyping procedure

a. Arraying template DNA

Use a liquid-handling system that is appropriate to the number of DNA samples to array. Decide whether to dry the DNA into the plate wells or use it in its hydrated form. Both approaches work well but have different advantages and disadvantages. DNA samples can also be arrayed manually if working with a small number of samples and large reaction volumes.

If small numbers of samples are to be genotyped, it is not worth drying the DNA samples. However, it is worth drying the samples if large numbers are to be genotyped in one run. Hydrated DNA arrayed in a PCR plate will quickly begin to evaporate differentially across the plate (plate edges evaporate more quickly than in the middle). Varying DNA volumes across the plate cause varying final reaction concentrations, leading to sub-optimal and much more varied genotyping results. For this reason, when drying the DNA into the plate wells the user must ensure that the DNA has dried to completion. Another reason to use dry DNA is reaction volume: if a very low 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 clean PCR plate, the PCR plate should be placed in a laboratory fan oven for one hour at 55˚C or until the samples have visibly dried. DNA dried into the plate should be stable long term at ambient temperature. When assembling the total reaction mix (see *Table 4*), water must be added in the correct proportion to account for the missing volume of the DNA template.

b. Reaction assembly

The PACE™ Genotyping Master Mix can be used with any reaction plate or well volume; there is no need to use a different product depending on reaction volume.
Note that total reaction volume shown in *Tables 3 & 4* are only approximate; the PACE™ genotyping assay mix volume is ignored as it does not cause any functionally meaningful dilution and doesn't affect the PACE™ Genotyping Master Mix performance.

It is very important that PACE™ Genotyping Master Mix is used at a final 1x concentration. $MgCl_2$ and DMSO can be used during troubleshooting of poorly performing assays with very low / high GC content respectively; however, they are not required in most cases.

	Hydrated DNA method (µL per well)		
	96-well plate	**384-well plate**	**384-well Array tape**
2x PACE GMM	5.0	2.5	0.8
PACE 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 GMM	5.0	2.5	0.5	0.4
PACE 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™ genotyping reactions must be thermally cycled according to the protocol described in *Table 5*.

Step	Description	Temp.	Time	N°. 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™ 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™ genotyping reactions.**

e. Fluorescent signal detection

After thermal cycling is complete, the fluorescent signal is detected by reading the plate in an appropriate fluorescent plate reader or qPCR machine in endpoint mode.

If sufficiently defined genotype clusters are not obtained after the initial thermal cycling protocol, the plate should be cycled for an additional three cycles using the conditions detailed in *Table 6* and read / analysed again. The additional cycling can be repeated until tight and well separated clusters are observed, though this is not often required.

f. Interpretation of data

Data analysis and interpretation can be done using cluster analysis software or alternatively can be carried out in Excel.

PACE™ Genotyping Master Mix reports genotypes with the fluorophores HEX and FAM. ROX passive reference dye can also be used to eliminate the effect of well-to-well liquid volume differences from the resultant cluster plot data. The inclusion of a passive reference leads to tighter clustering and, as a result, more accurate scoring of data (*Figure 1*).

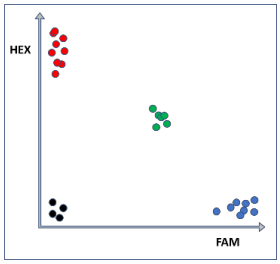

Figure 1. **Diagram of typical genotyping cluster plot data for the PACE™ genotyping chemistry. Normalization has been applied to the data on the right. Black samples at the origin are the no-template controls (NTCs).**

12. Ordering information

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

13. Licence information

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