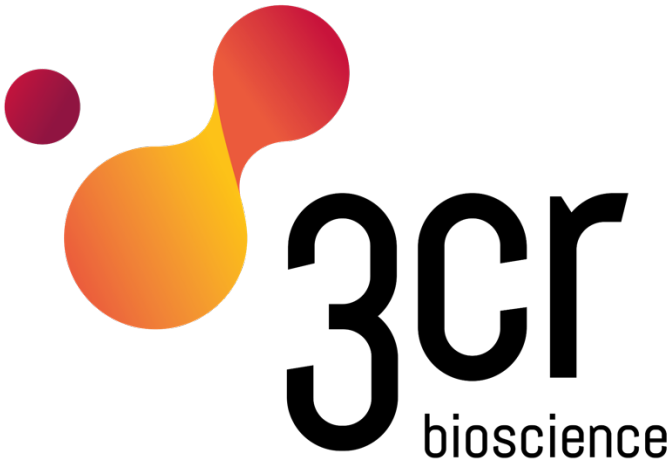

ProbeSure™ Multiplex Master Mix User Guide

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1. Product details

Product	Product volume	Part number	Reactions	Reaction volume	Concentration
ProbeSure™ Genotyping Master Mix (**No reference dye**)	2.5 mL	006-0001	500	10 µL	2x
	10 mL	006-0002	2,000	10 µL	2x
	25 mL	006-0003	5,000	10 µL	2x
	200 mL	006-0004	40,000	10 µL	2x
	1,000 mL	006-0005	200,000	10 µL	2x
ProbeSure™ Genotyping Master Mix (**with Atto633**)	2.5 mL	006-0006	500	10 µL	2x
	10 mL	006-0007	2,000	10 µL	2x
	25 mL	006-0008	5,000	10 µL	2x
	200 mL	006-0009	40,000	10 µL	2x
	1,000 mL	006-0010	200,000	10 µL	2x

2. Description

ProbeSure™ Multiplex Master Mix is designed for use in PCR genotyping applications with hydrolysis probe-based assays. It is specifically formulated for endpoint fluorescent detection of Single Nucleotide Polymorphisms (SNPs) and insertion/deletions (indels), or one reference gene with three further genes of interest and gives discrete genotyping clusters and high call rates for accurate and reproducible allelic discrimination.

ProbeSure™ Multiplex Master Mix can be used with a variety of fluorogenic probe chemistries including TaqMan®, ZEN™ probes and BHQ® / BHQ*plus*® probes. It is not intended for use as a general PCR master mix or for HRM applications.

ProbeSure™ Multiplex Master Mix contains all the necessary components to carry out the reaction (except DNA and primers / probes) and is supplied at 2x concentration. It is formulated with dTTP as this improves reaction sensitivity and efficiency when compared to mixes containing dUTP.

3. Storage and shelf life

ProbeSure™ Multiplex Master Mix is shipped on blue ice. Upon arrival, store at -20˚C; 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 be handled only 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

- ProbeSure™ Multiplex Master Mix (2x concentration)

Not included in the kit

- Fluorescent plate reader or qPCR machine
- PCR-grade water
- Primers and probes (or both combined into an assay)
- Template DNA.

6. ROX compatibility

ProbeSure™ Multiplex Master Mix is supplied either without Atto633 normalising dye or with Atto633 normalising dye. 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.

7. Primer and probe design

The optimal primer and probe concentrations should be determined empirically. Primer concentrations of 300 - 900 nM and probe concentrations of 100 - 200 nM are generally suitable for most applications.

8. DNA quality and quantity

ProbeSure™ Multiplex Master Mix can be used with purified DNA template (including cDNA) or crude extracts. We recommend using 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). Empirical optimization of DNA concentration by testing a sample dilution range is the most sensible approach. Such testing is particularly important when using crude lysates.

9. 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.

10. 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 since 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 causes variability in 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 1*), water must be added in the correct proportion to account for the missing volume of the DNA template.

b. Reaction assembly

Ensure the components are defrosted thoroughly; mix and centrifuge briefly prior to use. The enzymes used in ProbeSure™ Genotyping 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.

Component	Final Concentration	Volume for 10 µL reaction (µL)
ProbeSure GMM (2x)	1x	5
Primer(s)	300-900 nM	variable
Probe(s)	100-200 nM	variable
DNA template [1]	variable	variable
Nuclease-free water	-	To 10 µL
Total	**-**	**10 µL**

***Table 1*. Reagent volumes for total reaction mix.**
[1]Final concentration of cDNA 0.1 pg/µL -10 ng/µL; gDNA 10 pg/µL – 4 ng/µL

A total reaction mix should always be made to eliminate well-to-well variation of component concentrations. Assemble the total reaction mix using *Table 1* as a guide.

ProbeSure™ Multiplex Master Mix can be used in reaction volumes of any size, including volumes (below 1 µL) in the appropriate PCR plate. It is very important that ProbeSure™ Multiplex Master Mix is used at a final concentration of 1x.

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

Place the plate on a thermal cycler or qPCR instrument and carry out the thermal cycling step. The thermal cycling protocol used will vary with probe type but a guide to appropriate conditions is shown in *Table 2*.

Step	Description	Temp.	Time	Nº. Cycles
1	Enzyme activation	95˚C	10-15 min	1
2	Template denaturation	95˚C	10-15 secs	35-45
	Annealing and extension	57-65˚C	60 secs	

Table 2. **Thermal cycling guide for ProbeSure™ genotyping reactions**

e. Fluorescent signal detection

After thermal cycling is complete, the fluorescent signal is detected and assessed 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 3* and read / analysed again. The additional cycling can be repeated until tight and well separated clusters are observed, though this is rarely required.

Description	Temperature	Time	Nº. Cycles
Template denaturing	94˚C	10-15 secs	3
Annealing and extension	57-65˚C	60 secs	

Table 3. **Thermal cycling conditions for recycling ProbeSure™ genotyping reactions.**

f. Interpretation of data

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

Atto633 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*).

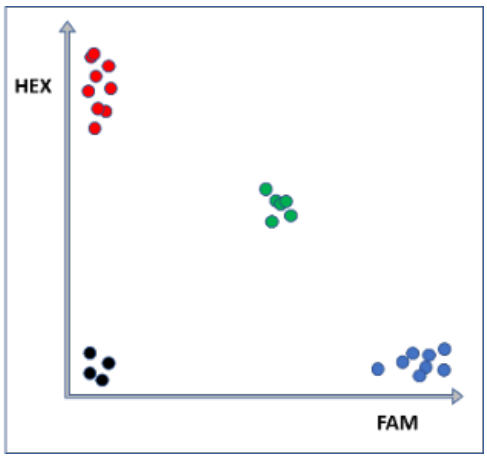

Figure 1. **Diagram of typical genotyping cluster plot data for the ProbeSure™ genotyping chemistry. The data shown (FAM and HEX fluorescence only) in the plot has Atto633 normalization applied. Black samples at the origin are the no-template controls (NTCs).**

11. Ordering information

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

12. Licence information

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