MACQUARIE UNIVERSITY ~ SYDNEY

BTF PRECISE MICROBIOLOGY

National Measurement Institute

Macquarie University Biotechnology Research Institute

Development of a Generic Inhibition Control for Real-Time PCR Assays

Leonardo B. Pinheiro (1), Moreland D. Gibbs (1), Graham Vesey (2), Zena Kassir (3), Kate R. Griffiths (3), Kerry R. Emslie (3) and Peter L. Bergquist (1)
(1) Macquarie University, Australia (2) BTF PTY LTD, Australia (3) National Measurement Institute, Australia

Summary

BTF Pty Ltd holds a proprietary technology for the production of BioBall™ for delivery of viable bacterial cells into microbiological media and test samples. It uses flow cytometry to dispense precise numbers of cells into a droplet of fluid, which is then frozen and freeze dried.

BTF's technology is being adapted to develop generic inhibition controls for real time PCR assays. The method involves generation of chromosomally-tagged bacterial strains with a single copy of a specific DNA sequence and incorporating it into the BioBall™ format. The freeze dried BioBall™ carrying precise numbers of tagged cells can then be used as quantitative positive controls for real time PCR.

The BioBall™ technology

1. Bacterial cultures are grown for maximum freeze drying recovery and viability.

2. A modified flow cytometer is used to count and sort bacteria into a single droplet (Fig 1).

3. The droplet is frozen in liquid nitrogen and placed into a vial.

4. BioBall™ are freeze dried, sealed under vacuum and crimped.

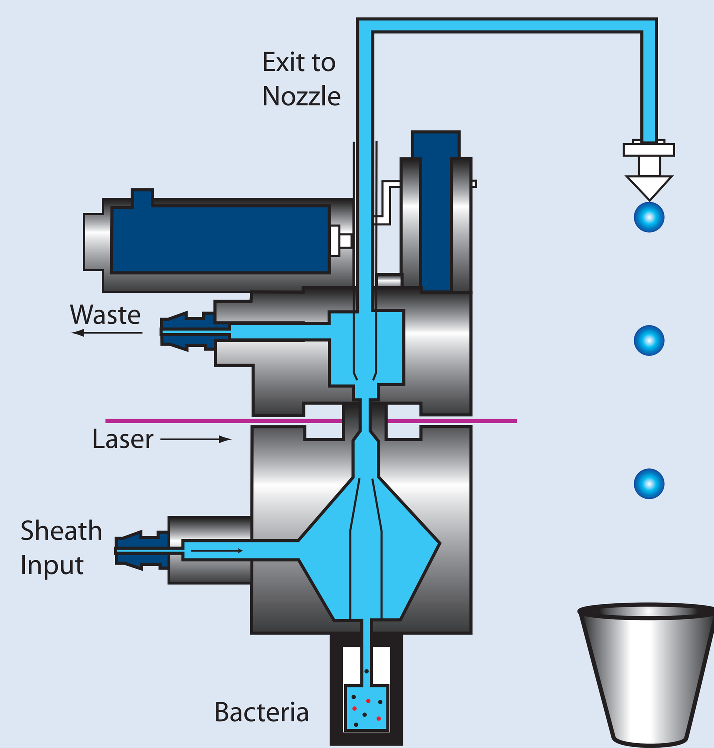


Fig 1: Bacteria are sorted and dispensed into liquid nitrogen by modified flow cytometry.

BioBall™ ready to use.

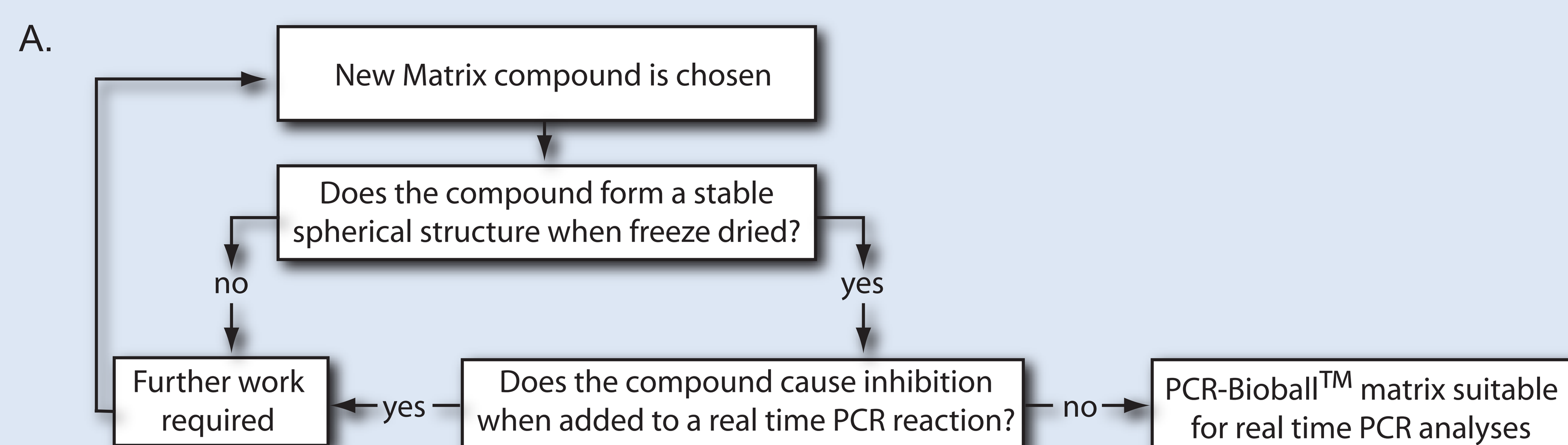
Plate out BioBall™ to obtain precise numbers of bacterial colonies

Add PCR-BioBall™ to real time PCR reactions to obtain precise number of copies of a specific target DNA sequence

Screening for a PCR compatible BioBall™ matrix

A number of alternative formulations were tested and optimized to obtain a matrix capable of forming a BioBall™ that does not inhibit PCR reactions (Fig 2).

A.



B.

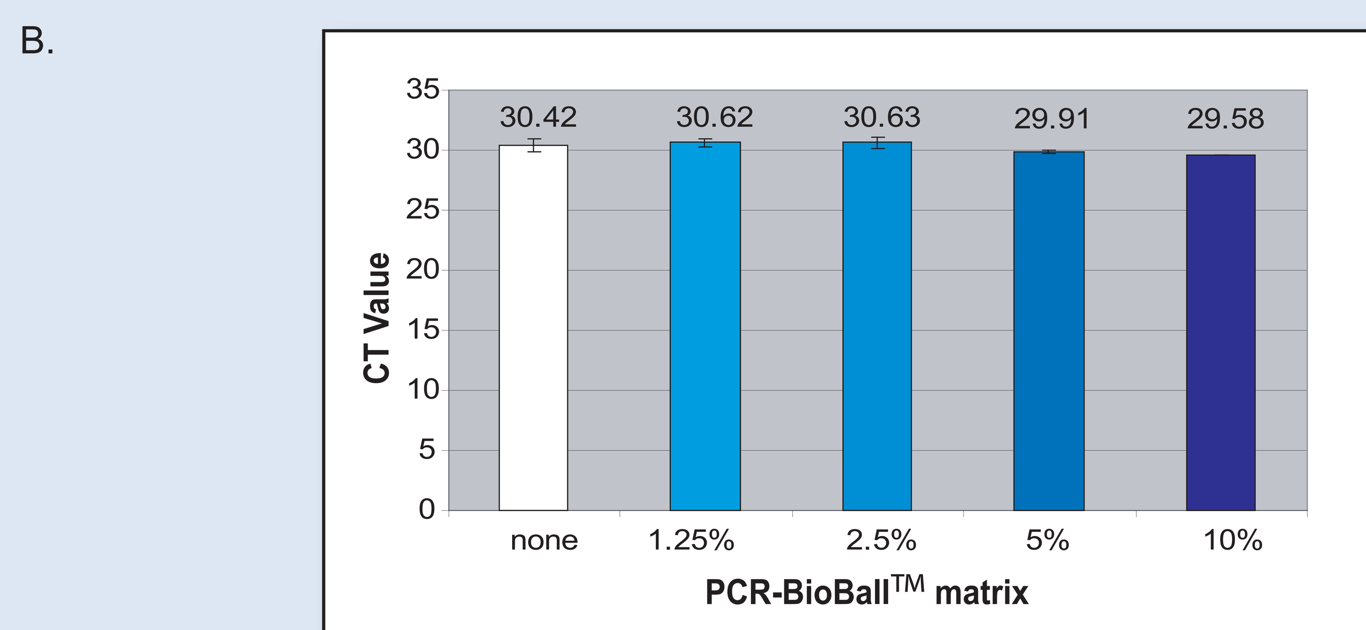


Fig 2: Strategy used for selection of a suitable PCR-BioBall™ matrix.
(A) Schematic diagram of the method use for matrix selection.
(B) Results from PCR assays using PCR-BioBall™ containing varying % of the selected matrix compound.

Generation of chromosomally-tagged bacterial cells

A transposition-based strategy was used for chromosomal integration of a target gene into bacterial cells.

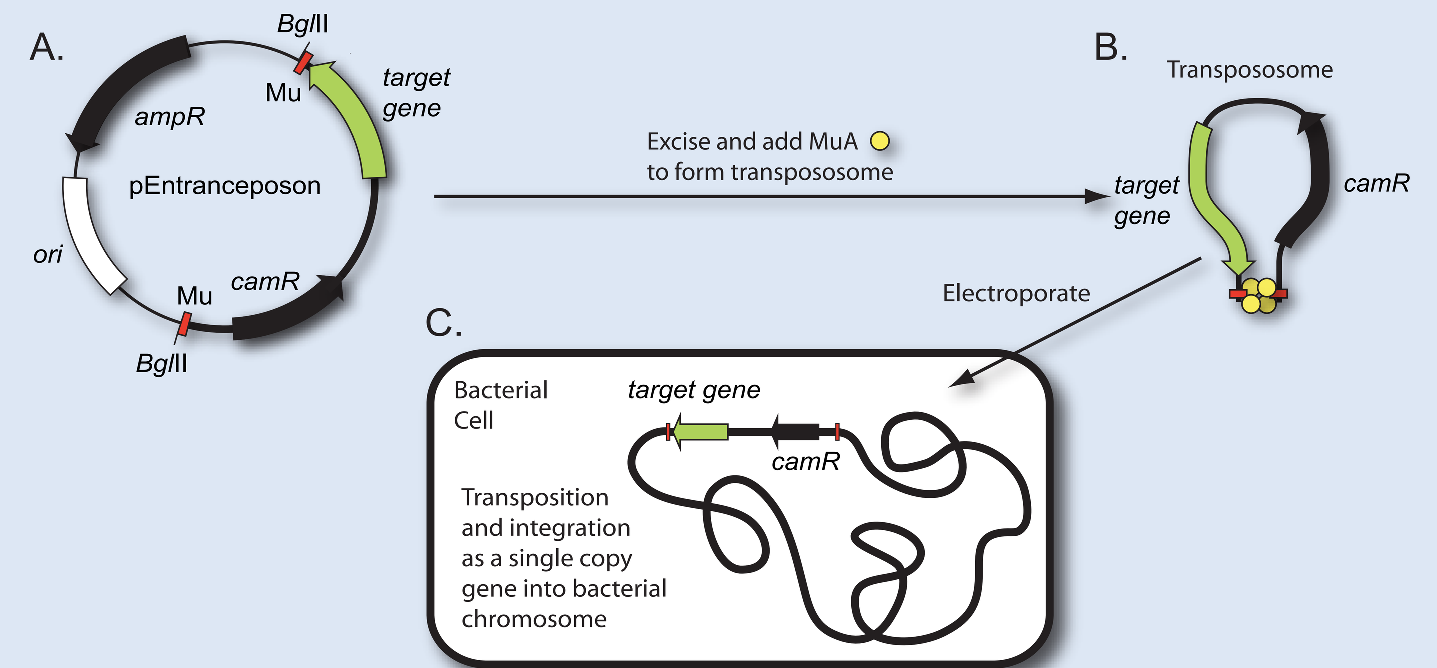


Fig 3: Transposition-based strategy used for chromosomal integration of a target gene into bacteria.
(A) The target gene transposition cassette is excised from plasmid vector.
(B) MuA transposase is added to excised transposon for *in vitro* assembly of transposition complexes called transpososomes.
(C) Assembled transpososomes are introduced into the cells by electroporation. Integration of the target gene as a single-copy was confirmed by Southern Blot analysis and the insertion point within the genome of the bacterium was determined by genomic walk PCR.

Real time PCR assays using PCR-BioBall™

Bacteria carrying an integrated single-copy of the target gene were incorporated into the PCR-BioBall™ format and used in real time PCR (Fig 4).

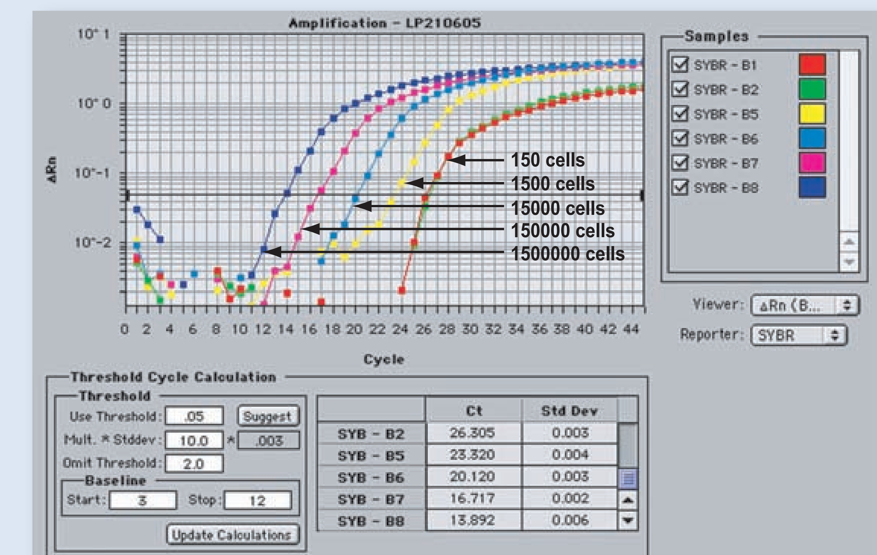
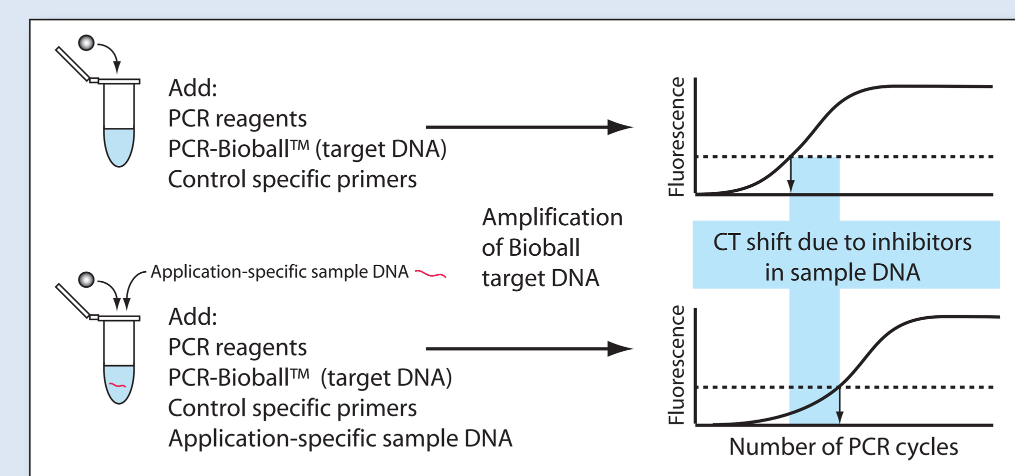


Fig 4: Results from PCR assays using PCR-BioBall™ containing defined numbers of bacteria with a single-copy chromosomally integrated target gene. Amplification of a 150 bp DNA segment of the target gene using SYBR green PCR master mix in an ABI prism 7700 SDS system.

PCR-BioBall™ as inhibition control for real time PCR assays



In its present format the PCR-BioBall™ inhibition controls have been designed to be added to duplicate PCR reactions. The control target sequence is amplified in the presence and absence of the application-specific sample DNA.

Fig 5: Use of PCR-BioBall™ as inhibition controls.

Under optimal conditions the CT value for the control should remain constant, since the reaction will have a fixed starting copy number of target DNA. In the presence of inhibitors the CT value will increase as amplification efficiency decreases (Fig 5). This change in CT value of controls can be used to normalise the CT value for the application specific targets.

Conclusions

BioBall™ technology is currently used as a reference standard for microbiological QC tests. This technology has been adapted for the development of precise copy number DNA controls for use as inhibition controls in real time PCR assays.

PCR-BioBall™ technology can be applied for the production of positive controls for PCR assays with very high accuracy and precision. These positive DNA controls can be used to quantify inhibition and then to normalise results of application-specific real time PCR. This normalisation significantly increases the accuracy of quantitative PCR analysis, particularly for low copy-number targets such as GM food testing at percentage threshold levels.

As well as inhibition controls, laboratories also use 'mimics' as positive controls by inserting application-specific target sequences into plasmid DNA. The PCR-BioBall™ format can also be tailored for the production of precise copy-numbers of application-specific positive controls by changing the target DNA sequence integrated into the bacterial genome.