Journal of Microbiological Methods 127 (2016) 111–122

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Influence of *Pichia pastoris* cellular material on polymerase chain reaction performance as a synthetic biology standard for genome monitoring

Alexander Templar, Stefan Woodhouse, Eli Keshavarz-Moore, Darren N. Nesbeth *

Department of Biochemical Engineering, University College London, Bernard Katz Building, London, WC1E 6BT, United Kingdom

ARTICLE INFO

Article history:
Received 29 October 2015
Received in revised form 9 May 2016
Accepted 16 May 2016
Available online 17 May 2016

Keywords:
Absolute quantitation
Synthetic Biology
Qpcr
Standardisation
Methylotrophic
Linear regression

ABSTRACT

Advances in synthetic genomics are now well underway in yeasts due to the low cost of synthetic DNA. These new capabilities also bring greater need for quantitating the presence, loss and rearrangement of loci within synthetic yeast genomes. Methods for achieving this will ideally; i) be robust to industrial settings, ii) adhere to a global standard and iii) be sufficiently rapid to enable at-line monitoring during cell growth. The methylotrophic yeast *Pichia pastoris* (*P. pastoris*) is increasingly used for industrial production of biotherapeutic proteins so we sought to answer the following questions for this particular yeast species. Is time-consuming DNA purification necessary to obtain accurate end-point polymerase chain reaction (e-pPCR) and quantitative PCR (qPCR) data? Can the novel linear regression of efficiency qPCR method (LRE qPCR), which has properties desirable in a synthetic biology standard, match the accuracy of conventional qPCR? Does cell cultivation scale influence PCR performance? To answer these questions we performed e-pPCR and qPCR in the presence and absence of cellular material disrupted by a mild 30s sonication procedure. The e-pPCR limit of detection (LOD) for a genomic target locus was 50 pg (4.91×10^3 copies) of purified genomic DNA (gDNA) but the presence of cellular material reduced this sensitivity sixfold to 300 pg gDNA (2.95×10^4 copies). LRE qPCR matched the accuracy of a conventional standard curve qPCR method. The presence of material from bioreactor cultivation of up to $OD_{600} = 80$ did not significantly compromise the accuracy of LRE qPCR. We conclude that a simple and rapid cell disruption step is sufficient to render *P. pastoris* samples of up to $OD_{600} = 80$ amenable to analysis using LRE qPCR which we propose as a synthetic biology standard.

1. Introduction

1.1. Synthetic biology and PCR

Much of the industrial exploitation of the methylotrophic yeast *Pichia pastoris* (*P. pastoris*) has been achieved by inserting transgenes into the host genome to high dosage (Inan et al., 2007). Synthetic biology can potentially increase this level of genetic modification by orders of magnitude through the construction and in vivo assembly of entire *Saccharmoyces cerevisiae* (*S. cerevisiae*) chromosomes arms (Dymond et al., 2011) and chromosomes (Annaluru et al., 2014). These chromosome-scale segments are typically refactored with the ability to undergo self-directed recombinative rearrangement to generate genotypically diverse populations. Disappearance or appearance of given sequences due to such rearrangements can, to an extent, be monitored using PCR-based methods.

In addition to monitoring target loci *per se*, it is also critical for industrial application of yeast synthetic biology that such methods are robust to industrial settings. Industrial application of yeasts frequently involves cultivation of cells to high cell density, placing cells under extreme recombinant protein synthesis burdens or tolerance of high concentrations of small molecule substrates or products. Such environments have a strong potential to exert selective pressure on cells to inactivate transgenes or synthetic genes by gene mutation, loss or rearrangement.

In addition to actual scale environments, high throughput, microscale screening methods are increasingly being used to isolate biological variants and conditions best suited to industrial application (Baboo et al., 2012). Such microscale approaches ideally mimic industrial conditions with respect to factors such as cell density. Monitoring synthetic yeast genomes during industrial scale processes could reveal any possible effects of selection pressure or locus instability exerted by a given production step or set of conditions. However, current options for locus quantitation involve relatively lengthy approaches such as Southern blotting or preparation of samples for qPCR in which DNA has been purified (Abad et al., 2010). These approaches tend not to be sufficiently

Abbreviations: PCR, polymerase chain reaction; BMGY, buffered glycerol complex medium; *e*-pPCR, end-point PCR; HCD, high cell density; qPCR, quantitative PCR; YPD, yeast extract peptone dextrose; SF, shake flask; wcw, wet cell weight; WCB, working cell bank; LRE, linear regression of efficiency; OCF, optical calibration factor.

* Corresponding author.
E-mail address: d.nesbeth@ucl.ac.uk (D.N. Nesbeth).

http://dx.doi.org/10.1016/j.mimet.2016.05.013

rapid to enable at-line monitoring of yeast cells within scale industrial processes or their scaled-down mimics.

Given the above it would be advantageous to develop PCR-based methods to quantify the abundance of sequence-specific DNA within yeast genomes, ideally with an absolute measurement standard and in a sufficiently rapid manner to enable at-line monitoring during an industrial process (actual or mimicked). In this study we address the timescales required to perform PCR by quantifying the extent to which sample processing is actually necessary to obtain accurate end point PCR (e-pPCR) and quantitative PCR (qPCR) data from high cell density *P. pastoris*.

To render biology more amenable to engineering approaches is a major aim of synthetic biology. Central to this aim is the establishment of standards for measurement of the quantities and activities of biomolecules. Relative qPCR, as the term suggests, yields only the fold difference in abundance of a target sequence relative to that of a reference target within an experimental sample (see Pabinger et al., 2014 for review). Absolute qPCR, by contrast, provides an absolute determination of the number of molecules of a given target sequence present within a sample so is a potentially much more powerful technique with respect to furthering the *biologicum machinalis* imperative.

The recently-developed linear regression of efficiency (LRE) method for absolute qPCR features a reportedly universal standard reference reaction (Rutledge and Stewart, 2010). The LRE qPCR method dispenses with two major elements of conventional absolute qPCR: the requirement for a target-specific standard curve (SC) and the use of crossing threshold values (Cts), both of which are discussed in depth in the excellent review by Kubista et al. (2006). Instead, for LRE qPCR the target quantity is inferred from applying a different statistical framework to SC qPCR and by applying this framework directly to fluorescence data and not to Ct values. Rutledge and Stewart (2010) tested 13 different primer pairs and purified template sequences to validate the LRE method and the putatively universal CAL1 standard reaction. To our knowledge LRE qPCR has not previously been used for analysis of target present in crude samples. To address the challenge of qPCR standardisation we seek to demonstrate the equivalence of LRE qPCR to the conventional standard curve (SC) qPCR method and propose LRE qPCR as a synthetic biology standard for qPCR in *P. pastoris*.

1.2. Standardisation enables industrial scaleup of synthetic biology

Historically, organisms such as yeast are treated by biotechnologists as complex natural phenomena that can be genetically modified by gene insertions to effect sufficient product yield at an acceptable degree of reproducibility and predictability. The emerging synthetic biology approach is for host genomes to be wholly defined and designed in order to achieve complete control over reproducibility, predictability and product yield. Developing methods that give comparable results across multiple instruments and laboratories is a key step in achieving this (Kelly et al., 2009, Müller and Arndt, 2012). Successfully establishing industrially robust synthetic biology standards is also likely to be beneficial for demonstrating regulatory compliance (Kaiser et al., 2008), which typically requires full compositional analysis of biological material throughout all the steps of an industrial process.

1.3. Industrial application of P. pastoris

P. pastoris has a track record of expressing hundreds of different recombinant proteins and has emerged as an effective microbial cell factory for production of biotherapeutics (Cereghino and Cregg, 2000; Macauley-Patrick et al., 2005) and bespoke fine chemicals (Zhang and Wang, 1994). *P. pastoris* brings inherent biological advantages to this role, such as amenability to genetic modification (Cregg et al., 2000), capacity for human-compatible post translational modification (Hamilton et al., 2013), low levels of endogenous protein secretion, high level secretion of exogenous proteins, high levels of intracellular enzyme expression (Yin et al., 2012) and rapid growth (Cos et al., 2006) to high cell density (HCD). Challenges for *P. pastoris* bioprocessing include mitigation of the impacts of HCD cultivation on cell physiology (Heyland et al., 2010) and the downstream challenges posed by high-solids yeast cell suspensions, such as dewatering (Lopes and Keshavarz-Moore, 2012) and clarification (Tolner et al., 2006).

1.4. Using PCR to quantify a target sequence during industrial cultivation

PCR is a highly sensitive and accurate method for quantifying levels of sequence-specific nucleic acids (Mackay, 2004). PCR-based assays have found utility in the measurement of genetic drift in process organisms (Foley and Shuler, 2010), barcoding industrial host cells (Parodi et al., 2002), mapping behaviour of microbial community structures (Tolvanen et al., 2008) and synthetic consortia (Bernstein and Carlson, 2012) and analysis of plasmid copy number during bacterial fermentation (Lee et al., 2006).

PCR-based assays often involve time-consuming multi-step sample purification procedures that can introduce experimental error and hamper assay throughput time (Lantz et al., 1994). Such lengthy sample preparation procedures tend to restrict the use of PCR to off-line, end point assays for contaminant detection. Establishing qPCR as a tool for at-line monitoring of cells within an industrial process would require significant reduction of sample preparation and reaction time. Designing PCR-based assay measurements to be quantitative in absolute terms by calibration against a standard would also enable direct comparison between different facilities and unit operation configurations.

1.5. Hypotheses tested in this study

Previous related studies tend to investigate the effects of different sample preparation methods (Olson and Morrow, 2012) or inhibitors (Plieskatt et al., 2014) on quantification of target sequences in foodstuffs (Sovová et al., 2016), clinical (Plieskatt et al., 2014) or environmental (Green and Field, 2012) samples by relative qPCR. These studies typically require DNA purification away from human cells or tissues or from bacterial cells. To our knowledge no studies have quantified the influence of bioreactor-derived material on absolute qPCR or e-pPCR for *P. pastoris*. Here we test the hypothesis that the presence of non-DNA cellular material does not compromise PCR accuracy in all cases. Specifically we are concerned to test if lengthy sample preparation procedures are necessary for accurate PCR-based assays of *P. pastoris* cellular material. We measure the influence of cellular material on the sensitivity of e-pPCR when used to detect the presence of a specific genomic sequence in a sample.

We also measure the influence of *P. pastoris* cellular material on performance of absolute qPCR for target quantitation. We test the hypothesis that the concentration and provenance of non-DNA cellular material are factors that influence qPCR accuracy. To test this we perform qPCR in the presence and absence of disrupted cells derived from high cell density cultivation in bioreactors and lower cell density cultivation in shake flasks. Finally, we test the hypothesis that LRE qPCR can match the accuracy of SC qPCR in the above conditions. We also discuss the suitability of LRE qPCR as a synthetic biology standard.

2. Materials and methods

All reagents were of molecular biology grade unless otherwise stated. All solutions were prepared using molecular biology grade water (Millipore, Billerica, USA). Oligonucleotides were synthesised by Eurofins MWG Operon (Acton, UK, www.eurofinsdna.com).

2.1. Cultivation of P. pastoris

The production strain used was *P. pastoris* GS115 (Invitrogen) expressing a recombinant human placental alkaline phosphatase under

the control of the methanol-inducible P_{AOX1} promoter. A single colony from a yeast-peptone-dextrose (YPD) agar plate (1% w/v yeast extract and peptone, dextrose, and agar all at 2% w/v) was used to inoculate 50 mL buffered glycerol complex medium (BMGY) broth (1% w/v yeast extract, 2% w/v peptone, 100 mM potassium phosphate pH 6, 1.34% w/v yeast nitrogen without amino acids, 1% v/v glycerol, and 0.4 μg/mL biotin) in a 250 mL shake flask which was incubated at 30 °C, with 250 RPM agitation for 15 h, after which typically $OD_{600} = 2$ was reached. This inoculum was used for a working cell bank (WCB) of over twenty 1 mL vials stored at −80 °C each containing 500 μL inoculum mixed with 300 μL 80% v/v glycerol.

For shake flask cultivation prior to fermentation, a WCB vial was thawed on ice and 100 μL of the glycerol stock solution used to inoculate 100 mL BMGY in a 500 mL shake flask before incubation at 30 °C, with 250 RPM agitation until $OD_{600} = 50$ was reached. 18 mL of this inoculum was used to inoculate 550 mL Basal Salt Media (BSM) in an Infors Multifors 1 L bioreactor (Infors UK Ltd., Reigate, Surrey, UK). BSM consisted of 26.7 mL 85% w/v H_2PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4 \cdot 7H_2O$, 4.13 g KOH, 40 g glycerol and 12 mL 'Pichia Trace Metal 1' (PTM1) solution (6.0 g/L $CuSO_45H_2O$, 0.08 g/L Nal, 3.0 g/L $MnSO_4 \cdot H_2O$, 0.2 g/L $Na_2MoO_4 \cdot 2H_2O$, 0.02 g/L H_3BO_3, 0.5 g/L $CoCl_2$, 20.0 g/L $ZnCl_2$, 65.0 g/L $FeSO_4 \cdot 7H_2O$, 0.2 g/L biotin, and 5.0 mL/L 96% H_2SO_4) per litre dH_2O. Bioreactor cultivation was performed according to a commercial protocol (Invitrogen, 2002). Agitation as impeller revolutions per minute (RPM) and dissolved oxygen tension (DOT) was recorded continuously.

We cultivated the *P. pastoris* recombinant protein production strain in complex medium in shake flasks and took a sample when $OD_{600} = 50$ for use as PCR template material (Fig. 1A). This optical density is typical of both the early stages of industrial scale cultivation and the dilution steps that can be necessary for efficient dewatering of high cell density *P. pastoris* cultures (Lopes and Keshavarz-Moore, 2012). Shake flask material was used to inoculate a 550 mL of BSM media in an Infors Multifors 1 L bioreactor. When $OD_{600} = 800$ was achieved, typically after 60–70 h of methanol-induction (Fig. 1B), a further sample was taken for PCR analysis. This represents a stage of cultivation when it is critical that recombinant protein yield and quality objectives have been met prior to harvest.

2.2. Total nucleic acid purification from samples

A modified version the the Harju et al. (2004) 'Bust 'n' Grab' method, described briefly below, was used to purify DNA from in the shake flask (Fig. 1A) and bioreactor (Fig. 1B) samples to determine typical DNA yields by spectrophotometry. After this, the volume of sample, ranging from 400 μL-8 mL, required to provide the DNA concentration in the undiluted template reactions indicated in Figs. 2 and 3 was centrifuged at 10,000 RPM for 3 min, re-suspended in 400 μL cell lysis buffer (2% Triton-×100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA) and freeze-thawed twice by incubating at −80 °C for 3 min and 95 °C for 1 min. Nucleic acid was extracted from cell lysate using standard phenol/ethanol extraction and purified DNA was resuspended in 400 μL 10 mM Tris (pH 7.5), which was then split into six aliquots of equal volume and stored at −20 °C. A given aliquot was thawed once for experimentation and any unused portion of the aliquot discarded.

2.3. Mild sonication to disrupt cells

Shake flask (Fig. 1A) and bioreactor (Fig. 1B) samples were sonicated using the procedure below to determine typical spectrophotometric and densitometric DNA estimations. Informed by these estimations, the volume of sample, from 400 μL-4 mL, required to provide the estimated DNA concentration in the undiluted template reactions indicated in Figs. 2 and 3, was centrifuged and re-suspended in dH_2O to a total volume of 400 μL. A Soniprep 150 sonicator (MSE, London, UK) was used to subject samples to a 10 second cycle of 100% amplitude sonication, followed by 10 s rest, three times. Total procedure duration was 5 ± 2 minutes (n = 20).

2.4. DNA mass estimation by spectrophotometry

DNA concentration was determined by spectrophotometry, using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The molar absorption coefficient of DNA and sample dilution factor were used to infer DNA concentration (Efiok et al., 2000). Shake flask process samples were analysed undiluted, and diluted 10 and 100 fold, and DNA mass at higher dilutions was then extrapolated from these

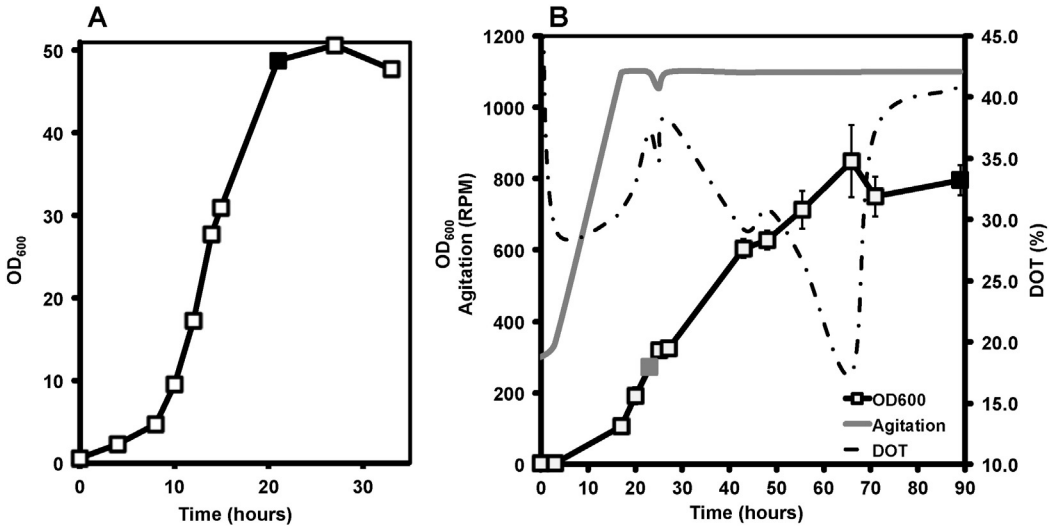

Fig. 1. Shake flask and high cell density cultivation of *P. pastoris* production strain GS115. A) A methanol-inducible GS115 *P. pastoris* production strain was used to inoculate 100 mL BMGY media in a 0.5 L shake-flask and an $OD_{600} = 50$ uninduced sample taken of cells entering stationary phase growth (filled square). Optical density at a wavelength of 600 nm (OD_{600}) was recorded at the indicated time points. The data set indicated is a representative experiment of n = 3 experimental repeats. B) 18 mL of shake flask culture was then used to inoculate 550 mL BSM media in an Infors Multifors 1 L bioreactor. 24 hours post-inoculation methanol was added for induction of transgene expression and an $OD_{600} = 800$ sample for PCR analysis was taken 66 hours post-induction (filled square). Data sets are representative of n = 3 experimental repeats. For the optical density measurements, error bars indicate standard deviation over n = 3 experimental repeats. Agitation as impeller revolutions per minute (RPM) and dissolved oxygen tension (DOT) was recorded continuously.

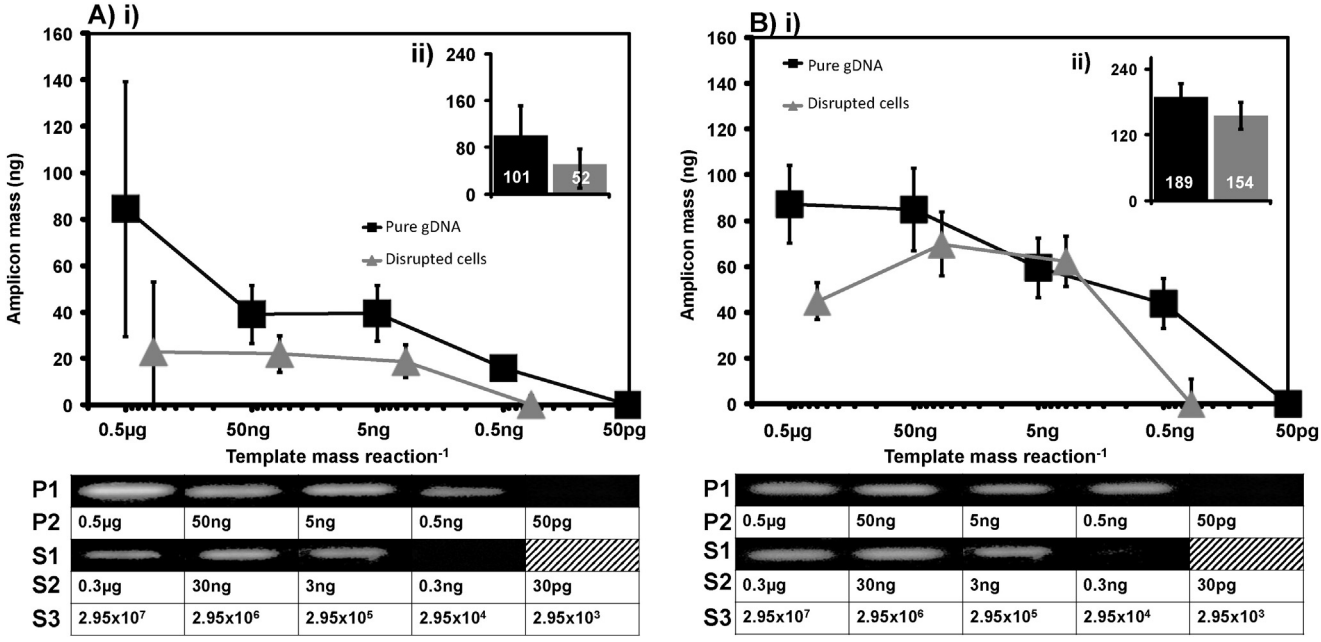

Fig. 2. Influence of disrupted cells on e-pPCR sensitivity. PCR and subsequent gel electrophoresis was performed using disrupted cells (grey symbols and lines) from shake flask (A) and bioreactor (B) cultivation as template material. Cell samples in which total gDNA had been purified away from cellular material were also used as template (black symbols and lines). For panels A and B, graph i) indicates densitometry measurements of resultant 104 bp amplicon band. Inlaid graph ii) plots the area under each curve in graph i) with the total value indicated in white text within the bar. For reactions with pure DNA as template, gel photos (row P1) and estimated template mass (row P2) are shown. For reactions in which disrupted cells were used as template, gel photos (row S1), estimated template mass (row S2) and estimated cell numbers present pre-sonication (row S3) are shown. Error bars indicate standard deviation over n = 3 biological repeats.

analyses. When analysing bioreactor process samples, it was found that DNA mass from samples at OD_{600} = 800 could not be accurately quantified, so 10, 100 and 1000 fold dilutions were analysed (Fig. 4).

2.5. DNA mass estimation by densitometry

Samples were run on 1% agarose gels stained with ethidium bromide and bands visualised using a GelDoc 2000 device (BioRad, Hercules, CA, USA) and Quantity One software version 4.6.8 (BioRad, Hercules, CA, USA). ImageJ software (version 1.46r, National Institutes of Health, Bethesda, USA) was used to select a region of the gel image, either a lane or a band, containing a known mass of DNA and a brightness value captured. A selected region of the same size and shape was then used to capture brightness of a region of unknown DNA concentration on the same gel. Background noise was subtracted using the 'Background Subtract' function provided by the ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2014).

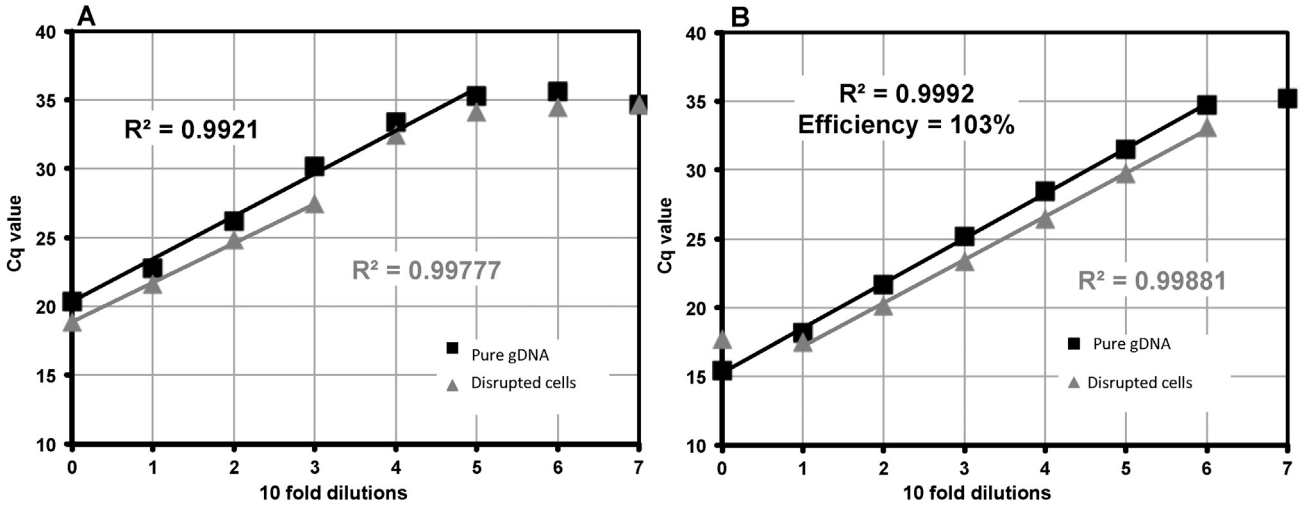

Fig. 3. Influence of disrupted cells on qPCR efficiency. Cq values were plotted as a function of tenfold dilutions of template material. Black square symbols indicate data points for pure gDNA template and grey triangles indicate data points when the template material was disrupted cells. Least-square fitting of a linear function was applied iteratively to the data points until all points of 100 ± 10% efficiency at R^2 = 0.99 were identified. These are indicated by the black lines between data points for pure gDNA template and grey lines between data points when the template material was disrupted cells. A) For shake flask material the undiluted cell sonicate sample from OD_{600} = 50 cultivation contained 776.1 ng DNA and the purified DNA sample contained 310 ng DNA. Amplification efficiency was within the defined limit for 6 data points for pure gDNA template and 4 data points for disrupted cell template. B) For bioreactor material the undiluted cell sonicate sample from OD_{600} = 800 cultivation was estimated to contain 2729 ng DNA and the purified DNA sample contained 1741 ng DNA. Amplification efficiency was within the defined limit for 7 data points for pure gDNA template and 6 data points for disrupted cell template. These profiles were observed over n = 3 technical repeats.

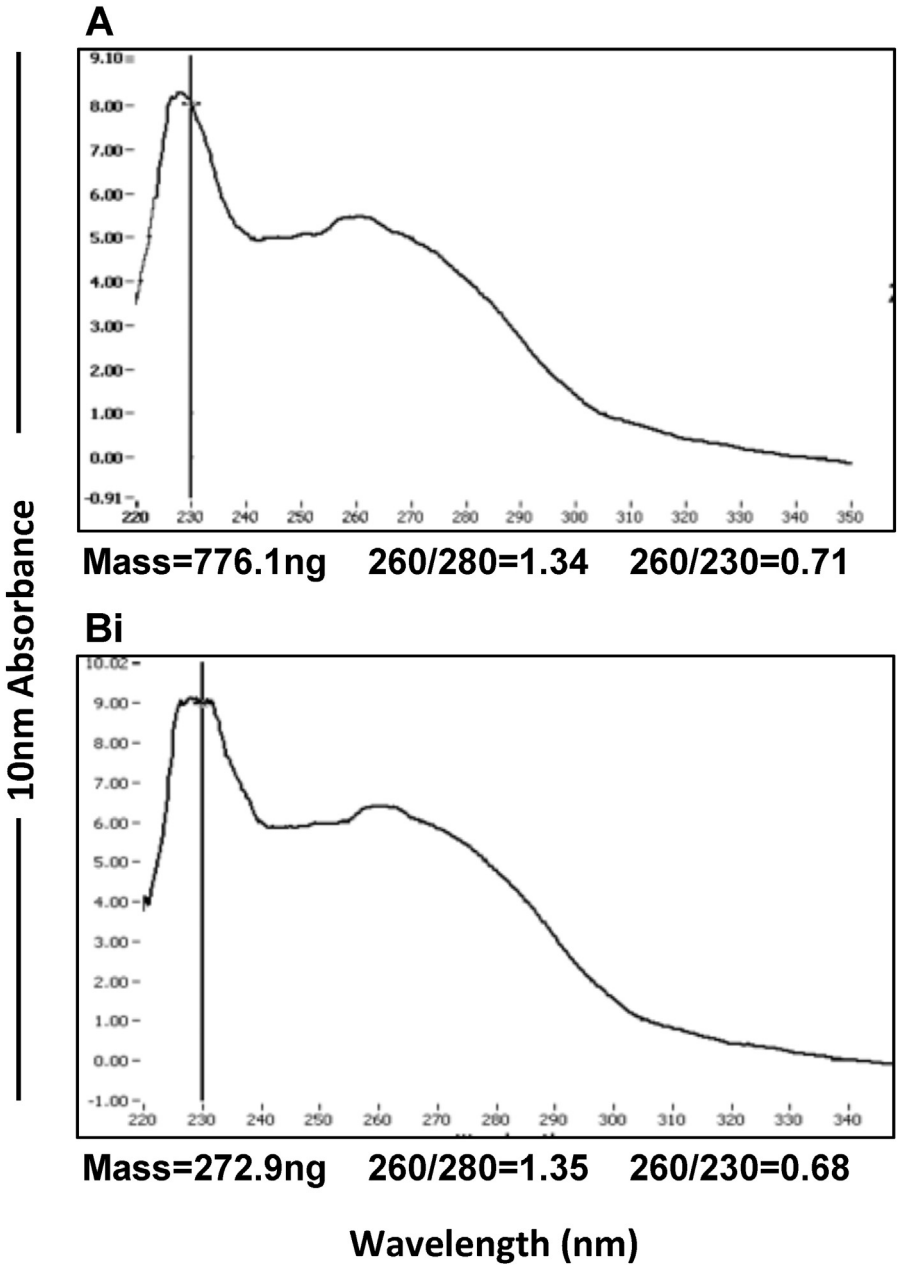

Fig. 4. Spectrophotometric measurements using disrupted cell suspensions. DNA absorbance profiles of disrupted cells derived from an $OD_{600} = 50$ shake flask sample (A) and a bioreactor sample tenfold diluted to $OD_{600} = 80$ (B) using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Data sets are representative of n = 3 biological repeats.

2.6. Primer design

Experimental primer sequences (Table 1) were designed in accordance with minimum information for publication of quantitative real-time PCR experiments (MIQE) standard guidelines (Mackay, 2004) and also refined for specificity with the National Center for Biotechnology Information (NCBI) 'Primer-blast' tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed 23.11.14) and for self-annealing with the NCBI 'PCR primer stats' tool (http://www.bioinformatics.org/sms2/pcr_primer_stats.html, accessed 23.11.14). CAL1 (Rutledge and Stewart, 2010) LRE qPCR primer sequences were used for calibration of data analysed by linear regression.

2.7. End-point PCR

Reactions were performed in a total volume of 50 μL, consisting of 5 μL 10× $MgCl_2$ polymerase buffer (100 mM Tris/HCl, 15 mM $MgCl_2$, 500 mM KCl), 0.5 μL Taq polymerase, 1 μL 10 mM dNTP (Sigma Aldrich,

Table 1
PCR primer sequences used in this study.

Target	Forward primer	Reverse primer
PAS_chr1-4_0150	TGGTGTCAGAGAGCATGGTA	CGTAGGACACGAAGTTCAGG
Lambda CAL1 region	AGACGAATGCCAGGTCATCTGAAACAG	CTTTTGCTCTGCGATGCTGATACCG

St. Louis, MO, USA), 1 μL each of forward and reverse primer stocks to give a final concentration of 500 nM for each, 5 μL of cell solution sample and water to final volume. A Veriti 96 well thermocycler (Applied Biosystems Grand Island, NY, USA) was used with cover heated to 105 °C. Each PCR was run for 40 cycles of: 95 °C for 5 s, 57 °C for 5 s, 72 °C for 30 s. The area under the curve, for which template was present in both sample types, was calculated using the trapezoidal method (Foley and Shuler, 2010). For 10 samples the total time taken for reaction assembly and device loading was 30 ± 10 (n = 8), 90 min for 40 cycles (no significant variation) and 45 ± 10 min for data capture by fluorescent agarose gel densitometry (n = 8).

2.8. Quantitative PCR assembly and real-time data capture

Reactions were carried out in a total volume of 20 μL, with each reaction containing 10 μL 2× SsoAdvanced (BioRad, Hercules, CA, USA) SYBR Green Supermix, 1 μL each of 10 μM forward and reverse primers, 5 μL of cell solution sample and water to final volume. Reactions were performed in a CFX Connect Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) with cover heated to 105 °C. Each PCR was run for 40 cycles using the same cycling conditions as above. Cq values were generated using Bio-Rad CFX manager 3.0 (BioRad, Hercules, CA, USA) and exported to Microsoft Excel 2010 for analysis. For 10 samples (n = 20), 40 cycles took 90 min (no significant variation) and real-time fluorescent data capture was concurrent.

2.9. Determination of amplicon production efficiency

Efficiency of qPCR reaction was calculated from a standard curve constructed from purified DNA samples that were serially diluted from neat stock and amplified by qPCR in parallel. Cq values from amplification curves were generated by the fit-point method and plotted against log dilutions. A straight line was drawn between data points corresponding to a coefficient of determination (R2) of 0.99. Linear regression was then applied to calculate efficiency (E), with the equation:

$$E = 10^{\left(\frac{-1}{slope}\right)}$$

2.10. Determination of copies of target DNA by 'standard curve' qPCR

The standard curve generated as described above was used to estimate copies of target in cell sonicate samples. Cq values of contaminated samples were plotted along the standard curve and converted into copy number using the equation:

$$targetcopynumber = 10^{\left(\frac{Cq-b}{m}\right)}$$

where b is the y-intercept and m is the slope of the standard curve.

2.11. Determination of copies of target DNA by 'LRE' qPCR

LRE qPCR, as described by Rutledge and Cote (2003), was also applied to measure copy numbers. LRE analyser v. 0.97 (Rutledge, 2011) was used according to developer's instructions. CAL1 primers and lambda DNA was used for calibration.

2.12. Statistical analyses

For a given sample, Bland-Altman analysis requires that the copy number of the target sequence determined by SC qPCR is deducted from the copy number determined by LRE qPCR (on the y axis) and this difference is plotted as a function of the mean of the two measurements (x axis). The average difference for all measurements is also calculated and plotted as the mean bias (dark dotted lines in Fig. 6, graphs B and D) across the data set. The standard deviation (±) of the mean bias is then multiplied by a factor of 1.96 and also indicated as upper and lower limits (grey dotted lines in Fig. 6, graphs B and D).

3. Results

3.1. Influence of disrupted P. pastoris cells on e-pPCR sensitivity

We sought to quantify the degree to which the presence of *P. pastoris* cellular material influences the sensitivity of e-pPCR. To achieve this we used primers specific to the single copy genomic (Table 1) locus, PAS_chr1-4_0150, for production of a 104 bp amplicon. Franciosa et al. (1996) had previously used a brief sonication procedure to enable PCR without the need for DNA purification from bacteria so we reasoned that a mild sonication procedure would make gDNA accessible to PCR while total cellular material is also still present. In order to disrupt cells and liberate host gDNA whilst minimising gDNA shearing or degradation, we used a brief and mild sonication procedure (see Materials and Methods). Agarose gel electrophoresis showed that no discernible shearing of gDNA had occurred post sonication compared to pre-sonication samples (data not shown). To quantify the effect of total disrupted cellular material, e-pPCR was performed using either purified gDNA or disrupted cells as template. We defined the limit of detection (LOD) as the first tenfold dilution of template material which resulted in no detectable amplicon band after n = 3 experimental repeats. To allow direct comparison of the effects of cultivation method, DNA template mass and cell numbers were matched between shake flask and bioreactor samples (Fig. 2).

The presence of shake flask cellular material reduced the total production of amplicon band, summed from all reactions, by 48% (from 101 to 52 arbitrary units) compared to the purified, gDNA-only template (Fig. 2A). By contrast, cellular material from bioreactor cultivation (Fig. 2B) reduced template amplification by only 18% (from 189 to 154 arbitrary units) and a greater overall level of template amplification was observed when compared to shake-flask derived material.

The observed LOD was 50 pg for purified gDNA for both shake flask (Fig. 2A) and bioreactor (Fig. 2B) samples. This is equivalent to 4.91×10^3 genome copies based on an assumed genome size of 9.43 Mb (De Schutter et al., 2009). The presence of cellular material reduced e-pPCR sensitivity sixfold to 300 pg gDNA (2.95×10^4 genome copies) for both shake flask and bioreactor samples.

3.2. Influence of disrupted P. pastoris cells on qPCR amplification efficiency

A plethora of approaches to qPCR data analysis are used across many fields of science and engineering. However, a practice common to many methods is to calculate the efficiency of amplicon production as an indicator of accuracy (Ruijter et al., 2013). Typically a threshold of 100 ± 10% efficiency, at a confidence level of $R^2 > 0.99$ (Gil and Coetzer, 2004), must be satisfied for a data point to be considered accurate.

As the primers used for e-pPCR above are MIQE (Bustin et al., 2009) compliant we used them to compare qPCR efficiency of amplification when purified genomic DNA (gDNA) and disrupted cellular material are used as template (Fig. 3), as an indicator of the degree to which sample preparation is necessary in qPCR-based procedures. We defined limit of quantitation (LOQ) as that template dilution for which statistical confidence in an efficiency of 100 ± 10% falls below $R^2 = 0.99$. For purified gDNA analysis the samples indicated in Fig. 1 underwent total gDNA purification followed by resuspension in water. For disrupted cellular material, the samples indicated in Fig. 1 underwent centrifugation and suspension in water before sonication. All samples were then tenfold diluted as indicated in Fig. 3 and used as template for qPCR.

For shake flask samples (Fig. 3A), pure gDNA enabled 100 ± 10% amplification efficiency, with $R^2 > 0.99$, over 6 template dilutions. The presence of cellular material reduces this to 4 dilutions, decreasing the LOQ by two orders of magnitude. Surprisingly, the equivalent experiment

with bioreactor samples (Fig. 3B), revealed amplification of both pure gDNA and disrupted cell material as template at 100 ± 10% efficiency over 6 or more dilutions. Tenfold dilution of the initial $OD_{600} = 800$ bioreactor sample, down to $OD_{600} = 80$, is required, after which the presence of yeast cell material has little effect on assay performance.

3.3. Influence of disrupted P. pastoris cells on 'standard curve' and 'LRE' qPCR

Rutledge and Stewart (2008) demonstrated a novel approach to qPCR using a method of linear regression of efficiency (LRE) for analysis of fluorescence data. LRE qPCR also features what Rutledge and Stewart (2008) refer to as a universal standard reaction, the OCF, consisting of a defined 'CAL1' primer pair plus lambda phage DNA as template (Table 1). Rutledge and Stewart (2010) report that the CAL1 reaction exhibits near-ideal behaviour of 100% amplification efficiency, enabling highly accurate correlation of amplification performance with the appearance of fluorescence. This approach does not require any relatedness between the CAL1 standard reaction and the experimental reaction. The authors tested LRE qPCR with the CAL1 over a four-month period with no significant change in performance. We suggest these inherent properties of the CAL1 standard make it ideal for use as a synthetic biology standard for application of qPCR to *P. pastoris* samples. To validate LRE qPCR with the CAL1 as a synthetic biology standard we compared its accuracy with that of a conventional Standard Curve (SC) method of qPCR.

Shokere et al. (2009) showed that spectrophotometry and the standard curve qPCR (SC qPCR) provide comparable DNA concentration measurements when used with purified DNA samples. Counter to our expectations, we also observed that spectrophotometry could be used to measure DNA concentration even when used with crude suspensions of disrupted cells from samples of up to $OD_{600} = 80$, despite the presence of components likely to distort the absorbance spectra (see Table 2 and typical absorbance profiles shown in Fig. 4). As such we used spectrophotometry as a mechanistically distinct comparator method to assess the performance of both LRE qPCR and SC qPCR.

Spectrophotometrically determined DNA mass in disrupted cellular material and pure DNA samples (Fig. 4) was used to predict genome copy number based on a *P. pastoris* genome size of 9.43 Mb (De Schutter et al., 2009). As the target locus is known to be present as a single copy within the *P. pastoris* genome, genome copy number is assumed to be equivalent to target sequence copy number. As such we converted three spectrophotometer measurements to target sequence copy numbers (Table 2). We plotted these three spectrophotometrically derived target sequence copy numbers (grey circles in Fig. 5) as a function of template dilution and linearly extrapolated the trend (dashed lines) for both shake flask (Figs. 5A) and bioreactor material (Fig. 5B).

For the conventional SC qPCR method the 'standard curve' used for calibration is generated by linear regression of Cq values obtained with log dilutions of purified samples of template DNA (Fig. 5C). The template DNA used for this standard curve must be purified, of known concentration and also the same sequence as the DNA expected to be present in the experimental samples at unknown concentration. As such, purified gDNA samples whose concentration has been measured by spectrophotometry represents the standard curve for the SC qPCR method. For the LRE qPCR method the proposed CAL1 universal standard was used to calibrate the data and linear regression is applied directly to the fluorescence curve for every cycle of the reaction (Fig. 5D).

For shake flask material (Fig. 5A), use of the SC method enabled quantification of target DNA in undiluted ($OD_{600} = 50$) cellular material and over two further tenfold dilutions after which copy number values diverged from the spectrophotometric data. The LRE qPCR method produced results in agreement with spectrophotometric data for undiluted material but also over three further tenfold dilutions. For bioreactor material (Fig. 5B), both LRE qPCR and SC qPCR methods are in close agreement with spectrophotometrically derived target DNA copy number (Table 2) for material that has undergone one tenfold dilution (therefore $OD_{600} = 80$) and five further tenfold dilutions.

3.4. Statistical comparison of 'standard curve' and 'LRE' qPCR methods

We next used an XY plot (Burd, 2010) to compare SC qPCR and LRE qPCR for quantifying levels of target DNA in disrupted cellular material. A slope of 1.00 indicates zero bias between methods. XY plot showed that, for shake flask material, LRE qPCR (Fig. 6A) showed marginal proportional bias (slope of 0.914) of SC qPCR. The Y intercept for this comparison did show large deviation from zero (0.502), which would suggest real systemic bias of SC qPCR. However, Bland-Altman bias plots (Bland and Altman, 1986) indicated that LRE qPCR and SC qPCR methods are equivalent for analysis of shake flask derived samples (Fig. 6B), as the mean bias spanned the zero difference level (Burd, 2010).

For bioreactor-derived samples, an XY plot of SC qPCR and LRE qPCR data revealed a slope of 1.1758, indicative of only modest proportional bias (Fig. 6C). Although again the Y intercept for this comparison did show large deviation from zero (−0.8513), a Bland-Altman bias plots was consistent with the LRE qPCR and SC qPCR methods being equivalent as the mean bias level spanned zero difference (Fig. 6D).

3.5. LRE qPCR with CAL1 OCF as a synthetic biology standard for qPCR in P. pastoris

Unlike SC qPCR, LRE qPCR does not require that a standard curve that consists of the same primers and target as the experimental samples of unknown target DNA concentration. This allowed us to measure the profile of LRE qPCR accuracy for both purified gDNA and disrupted cellular material (Fig. 7). All LRE qPCR data in Fig. 7 was calibrated using the CAL1 primers and target. As previously, we used spectrophotometric data to predict genome copy number present in a given sample.

Shake flask data in Fig. 7A shows the effect of the presence of cell material from shake flask on the ability of LRE qPCR to match the spectrophotometric prediction, with agreement for undiluted material and over three subsequent tenfold dilutions. For HCD bioreactor material (Fig. 7B) LRE qPCR also matched spectrophotometric data across all samples except for the most concentrated sample and most dilute samples, neither of which yielded amplicon.

Table 2
Spectrophotometric DNA measurements for samples used in qPCR experiments and an indication of DNA loss in the purification step.

Source	Dilution	DNA (ng/μL) in disrupted cell solution	DNA (ng/μL) DNA purified from cells	DNA Loss (%)
Shake flask	0	776.1	319	58.9
	1	79.4	31	60.96
	2	7.7	3.2	59.09
			Mean	59.65%
Bioreactor	1	272.9	174.1	36.2
	2	30.9	17.4	43.69
	3	3.1	1.7	45.16
			Mean	41.68%

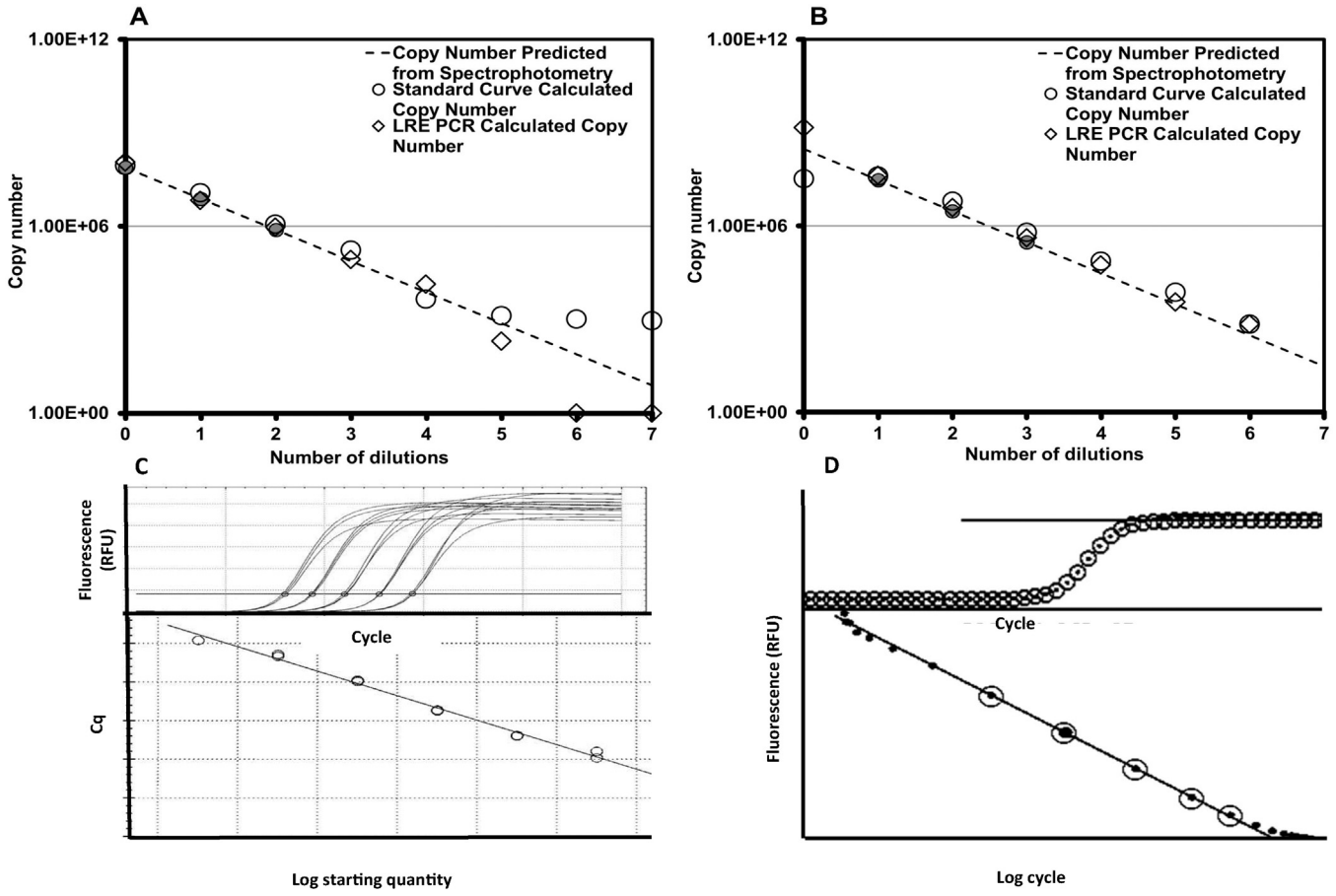

Fig. 5. Comparison of SC qPCR and LRE qPCR using disrupted cells as template. Both graph A (shake flask) and graph B (bioreactor) incorporate the following features. Grey data points indicate spectrophotometric data (the absorbance profiles for the disrupted cell sample derived from $OD_{600} = 50$ shake flask culture (A) and bioreactor culture diluted to $OD_{600} = 80$ (B) are shown in Fig. 4). Dashed lines linearly extrapolate the spectrophotometric data points to predict copy number at lower dilutions. Open circles indicate copy number of target present in disrupted cells as determined by SC qPCR. Open diamond symbols indicate copy number of target present in disrupted cells as determined by LRE qPCR. Graph C shows the standard curve used for SC qPCR quantification, formed by plotting the Cq values (open circles) of multiple reactions as a function of the log of their serial dilution. Raw fluorescence data and a horizontal 'crossing threshold' line is shown in the inlaid graph above the Cq data. In graph D fluorescence values (circles) of the CAL1 reaction are plotted against their cycle number (upper panel) and the log of their cycle number (lower panel). Data sets are representative of n = 3 biological repeats.

4. Discussion

Sample preparation is normally performed to remove inhibitors that might impact the accuracy and sensitivity of PCR-based assays (Dineva et al., 2007). Ionic detergents, phenol and metal salts that may be present in growth media can all inhibit PCR. Kits and reagents used to extract, purify and preserve DNA can also influence PCR and bring the risk of introducing error through loss of DNA (Miller et al., 1999), introduction of inhibitory biological material from disrupted cells and co-purification of chemical inhibitors (Schrader et al., 2012). Some DNA isolation kits have also been shown to produce false-positive results due to the presence of contaminant DNA in the kit, the level and source of which varies between manufacturer and batch (Queipo-Ortuño et al., 2008).

4.1. P. pastoris cellular material depresses e-pPCR sensitivity

End-point PCR remains a valuable tool for detecting contaminant organisms during scale up or storage of biological material. One factor that delimits e-pPCR application is the time it takes to perform sample preparation. We sought to measure the extent to which sample preparation is necessary for garnering a reliable yes/no binary datum using e-pPCR (Fig. 2). Cell suspensions from shake flask or bioreactor cultivation were sonicated for 30 s as part of a procedure that took a total of 5 min to perform before being used in e-pPCR.

As expected, significant variation in the level of amplicon production was observed between experimental repeats - underlining the fact that e-pPCR is best suited to detection and not quantitation. The total mass of amplicon produced was greater for bioreactor material than for shake flask material even though the templates had been matched in terms of number of cells and gDNA mass (see Materials and methods). Despite the difference in amplicon production, both shake flask and bioreactor material reduced e-pPCR sensitivity sixfold (Fig. 2). These observations suggest that *P. pastoris* sample preparation for binary e-pPCR assays is necessary to avoid a significant reduction in LOD.

4.2. Cellular material from P. pastoris bioreactor cultivation does not depress qPCR accuracy

In contrast to e-pPCR, qPCR is used widely to accurately quantify relative or absolute abundance of DNA. Polymerisation and analysis occur in parallel in most qPCR platforms, unlike e-pPCR in which gel electrophoresis and gel analysis are performed serially after PCR. Sample preparation therefore represents a greater proportion of total assay time (Fig. 8) for qPCR. As such we characterised the influence of shake flask and bioreactor samples on qPCR efficiency again using sonication to represent a non-processed sample in which DNA and all other cellular components remain present throughout polymerisation.

LRE qPCR is calibrated against the CAL1 reaction so we could directly test its performance using purified gDNA samples and disrupted cells as template. This allowed us to map the influence of shake flask and

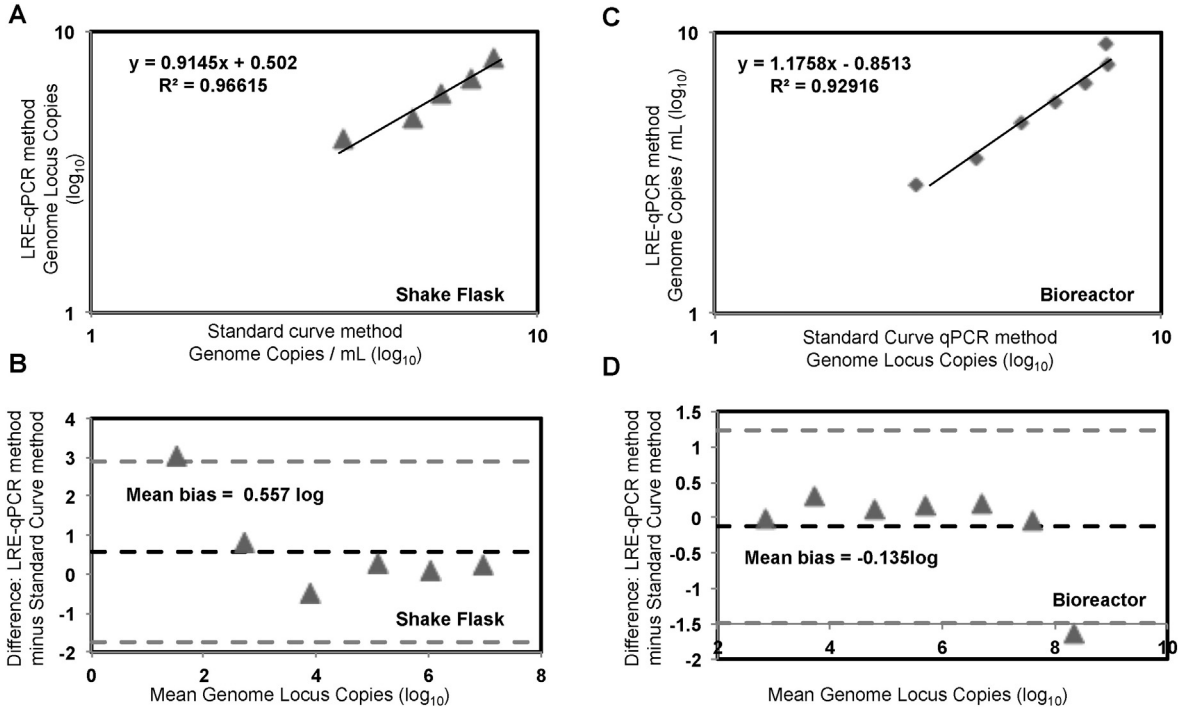

Fig. 6. Statistical comparison of SC qPCR and LRE qPCR. XY plots (graphs A and C) were derived from copy number estimations made using the indicated method, using data plotted in graphs A and B of Fig. 5. Bland-Altman bias plots (graphs B and D) were derived from XY plots. Statistical procedures were performed as described by Burd (2010). Mean bias (difference) is indicated by the dark dashed lines and 1.96× the standard deviation of this bias (±) is indicated by the grey dashed lines. Data sets are representative of n = 3 biological repeats.

bioreactor material on LRE qPCR analysis. Fig. 3A data shows that shake flask material does compromise the LOQ for LRE qPCR but Fig. 3B indicates that bioreactor material has minimal effect on LRE qPCR, except for the most concentrated (originating from OD_{600} = 800 material) and the most dilute samples.

4.3. Rapid and crude P. pastoris sample preparation can be sufficient for capturing accurate quantitation data with LRE qPCR

Commercial sample preparation kits, including membrane and bead-based systems, often involve protocols that comprise 20 steps or more and can take over one hour to perform. Our findings suggest that a simple and rapid (approximately 5 min) sonication procedure is sufficient to render HCD bioprocess samples amenable to LRE qPCR analysis. Current (http://www.roche.com/products/product-details.htm?type=product&id=64) and prototypical (www.xxpresspcr.com) ultra-rapid PCR devices offer the potential to reduce reaction and analysis time to <20 min. A 30 minute or less total procedure duration would pose the real possibility of LRE qPCR being used as an at-line bioprocess monitoring tool and as such offer new analytical power for process development. To illustrate this we logged the length of time taken to complete several of the procedures undertaken during this work and used these data to project likely future assay durations in Fig. 8.

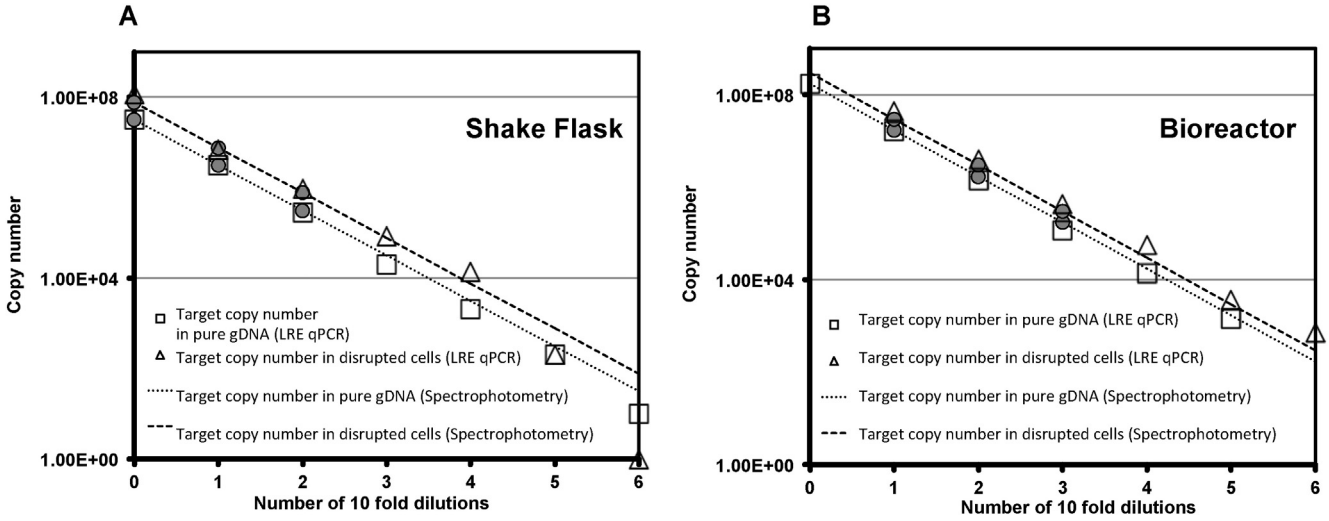

Fig. 7. LRE qPCR performance using pure gDNA and disrupted cells as template. LRE qPCR was performed on the same samples derived from shake flask (A) and bioreactor (B) cultures that were used in Fig. 3. Grey data points indicate spectrophotometric data and dashed lines extrapolate these data to predict copy number at lower dilutions. LRE qPCR data is plotted with square symbols for purified gDNA and triangles for disrupted cells. Data sets are representative of n = 3 biological repeats.

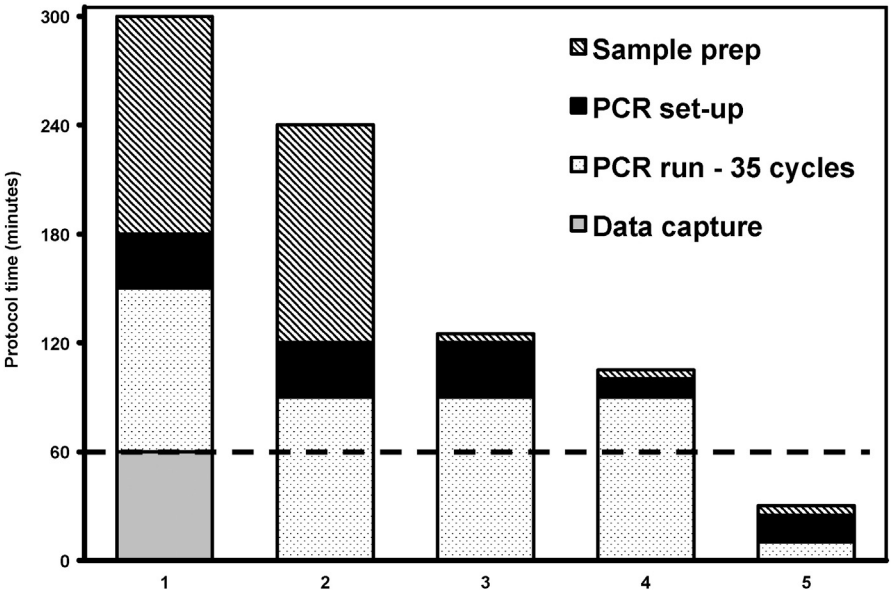

Fig. 8. Predicted and known time profiles of different PCR methods. X axis numbers indicate each method. Method 1 Endpoint PCR and agarose gel analysis. Genomic DNA sample preparation using the Qiagen DNEasy kit (Qiagen, 2006) took just under 2 h (n = 2), for 10 samples. PCR assembly and device loading was 30 ± 10 (n = 8), 90 min for 40 cycles (no variation) and 45 ± 10 min for data capture by agarose gel and fluorescence densitometry (n = 8). Method 2 Standard Curve qPCR using a BioRad CFX qPCR device with parallel fluorescent measurement and analysis. For 10 samples (n = 20), 40 cycles took 90 min and real-time fluorescent data capture was concurrent. Sample and reaction preparation times were the same as Method 1. Method 3 Same as Method 2 but sample preparation was a sonication step of 5 ± 2 min duration (n = 20). Method 4 LRE qPCR. Same as Method 3 but PCR assembly and device loading took less time, 15 ± 5 (n = 10), a no standard curve was required. Method 5 LRE qPCR with ultra-rapid device. Same as Method 4 but 40 cycle PCR run time predicted to be reduced to 10 min if a device such as the Xxpress (BJS Biotechnology) or the Roche LightCycler were used. A total duration of 60 min or less (dotted line) is compatible with at-line bioprocess monitoring.

4.4. P. pastoris cell provenance appears to influence DNA purification

Why does the presence of shake flask material affect both e-pPCR (Fig. 2) and qPCR (Figs. 3, 5 and 7) more than material derived from high cell density bioreactor cultivation? This may be due to the physiological status of cells 66 hours post-induction and at the end of idiophasic cultivation, compared to their pre-induced state during seed train, shake flask growth (Fig. 1). To maximise product yield many microbial fermentations typically proceed for durations that can cause significant physiological impact on cells (Sandén et al., 2003). Levels of dyshomeostasis and misfolded protein accumulation in late idiophase cells may have the net effect of making cellular material more readily unbind DNA than is the case for healthier cells. The observation that phenol/chloroform DNA purification from shake flask material is more lossy than purification from cells originating from high cell density bioreactor cultivation (Table 2, and plotted in Fig. 7), is consistent with this hypothesis.

4.5. LRE qPCR CAL1 reaction as a synthetic biology standard

Most current efforts in qPCR standards deal with experimental setup (Bustin et al., 2009), food (Malorny et al., 2003) or water safety testing, with several standards (Table 3) agreed by the International Standards Organisation (ISO). We suggest that, due to the advantages of the CAL1 OCF standard, LRE qPCR need only match the accuracy of conventional SC qPCR in order to be a credible standard for bioindustry and the synthetic biology community. Fig. 5 shows that, for both shake flask and bioreactor material, LRE qPCR matched SC qPCR in ability to confirm copy number predictions made by extrapolation of spectrophotometric data. Head-to-head comparison also showed the methods to be equivalent (Fig. 6).

Assay duration is a key delimiting factor at present in the application of qPCR for monitoring bioindustrial process streams. In future, assay duration is also likely to be a key factor when monitoring the status of performance-critical loci within synthetic yeast genomes or gene

Table 3
Comparison of calibration and standardisation for LRE and SC qPCR. Rutledge and Stewart (2010) observed negligible variation in the performance of the CAL1 OCF over 8 runs across a 4-month period. We interpret this observation as a strong indicator that calibration runs may only be necessary for LRE qPCR is infrequently as once every 4 months.

	Calibration method	Recommended frequency of calibration	Proposed standards
Standard curve	Parallel reactions of samples containing known DNA mass Higuchi et al. (1993).	Every assay Higuchi et al. (1993).	ISO 22119:2011 De Schutter et al. (2009)). ISO 22119:2011 De Schutter et al. (2009). ISO/TS 13136:2012. ISO/TS 12869:2012. ISO/TS 21569–2:2012. Standard proposed by Malorny et al. (2003).
LRE	OCF generated from the CAL1 lambda DNA calibration reaction.	Every 4 months Rutledge and Stewart (2010).	This report.

networks (Guo et al., 2015). A significant potential advantage LRE qPCR brings is the reduced need for calibration runs. The CAL1 reaction was reported by Rutledge and Stewart (2010) to perform consistenly throughout a 4 month period – suggesting the possibility that CAL1 calibration runs might only need to be performed at a frequency of one run every 4 months.

5. Conclusions

We have shown that sample preparation is a critical requirement for e-pPCR analysis of *P. pastoris* material from both shake flasks and bioreactors. By contrast, for qPCR analysis a simple and rapid sonication step, with no attempt at DNA purification, is sufficient to capture accurate qPCR data from HCD bioreactor material. However, it is likely that a degree of DNA purification is necessary for accurate qPCR analysis of shake flask-derived *P. pastoris* material.

LRE qPCR has inherent advantages in terms of standardisation and the frequency of required calibration reactions. LRE qPCR matches conventional Standard Curve qPCR with respect to absolute quantification of target DNA, even in the presence of $OD_{600} = 80$ material. Although not demonstrated here, we predict that in the near future a combination of rapid sample preparation, adoption of the LRE qPCR CAL1 standard and devices capable of ultra-rapid PCR, will enable expansion of qPCR to at-line monitoring of yeasts controlled by synthetic genomes at scale. We invite the synthetic biology and biotechnology communities to test further the CAL1/OCF standard and LRE qPCR method for absolute quantitation of genomic sequences in *P. pastoris* and to assess the procedure as a standard for use with other organisms.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by the EPSRC and BJS Biotechnologies Ltd (EP/G034656/1). DNN and AT conceived of the study, participated in its design and coordination and helped to draft the manuscript. AT contributed to cell cultivation, carried out all PCR studies, performed the statistical analysis and drafted the manuscript. SW carried out the cell cultivation and contributed to drafting of the manuscript. EKM participated in the design of the cell cultivation studies. All authors read and approved the final manuscript.

References

Abad, S., Kitz, K., Hörmann, A., Schreiner, U., Hartner, F.S., Glieder, A., 2010. Real-time PCR-based determination of gene copy numbers in *Pichia pastoris*. Biotechnol. J. 5, 413–420.

Annaluru, N., et al. Science. 344 (6179), 55–58. http://dx.doi.org/10.1126/science.1249252 Epub 2014 Mar 27. Erratum in: Science. 2014 May 23;344(6186):816.

Baboo, J.Z., Galman, J.L., Lye, G.J., Ward, J.M., Hailes, H.C., Micheletti, M., 2012. An automated microscale platform for evaluation and optimization of oxidative bioconversion processes. Biotechnol. Prog. 28, 392–405.

Bernstein, H.C., Carlson, R.P., 2012. Microbial consortia engineering for cellular factories: in vitro to in silico systems. Comput. Struct. Biotechnol. J. 3.

Bland, J.M., Altman, D.G., 1986. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet. 1 (8476), 307–310.

Burd, E.M., 2010. Validation of laboratory-developed molecular assays for infectious diseases. Clin. Microbiol. Rev. 23 (3), 550–576.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622.

Cereghino, J.L., Cregg, J.M., 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol. Rev. 24, 1.

Cos, O., Ramon, R., Montesinos, J., Valero, F., 2006. Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast Pichia Pastoris under different promoters: a review. Microb. Cell Factories 6, 5.

Cregg, J.M., Cereghino, J.L., Shi, J., Higgins, D.R., 2000. Recombinant protein expression in Pichia pastoris. Mol. Biotechnol. 16 (1), 23–52 Review.

De Schutter, K., Lin, Y.-C., Tiels, P., Van Hecke, A., Glinka, S., Weber-Lehmann, J., Rouzé, P., Van de Peer, Y., Callewaert, N., 2009. Genome sequence of the recombinant protein production host *Pichia pastoris*. Nat. Biotechnol. 27, 561–566.

Dineva, M.A., MahiLum-Tapay, L., Lee, H., 2007. Sample preparation: a challenge in the development of point-of-care nucleic acid-based assays for resource-limited settings. Analyst 132, 1193–1199.

Efiok, B.J.S., Eduok, E.E., International, A., 2000. Basic calculations for chemical and biological analysis. AOAC Int.

Foley, P.L., Shuler, M.L., 2010. Considerations for the design and construction of a synthetic platform cell for biotechnological applications. Biotechnol. Bioeng. 105, 26–36.

Franciosa, G., Fenicia, L., Caldiani, C., Aureli, P., 1996. PCR for detection of *Clostridium botulinum* type C in avian and environmental samples. J. Clin. Microbiol. 34, 882–885.

Gil, M.E., Coetzer, T.L., 2004. Real-time quantitative PCR of telomere length. Mol. Biotechnol. 27, 169–172.

Green, H.C., Field, K.G., 2012. Sensitive detection of sample interference in environmental qPCR. Water Res. 46, 3251–3260.

Guo, Y., Dong, J., Zhou, T., Auxillos, J., Li, T., Zhang, W., Wang, L., Shen, Y., Luo, Y., Zheng, Y., Lin, J., Chen, G.Q., Wu, Q., Cai, Y., Dai, J., 2015. YeastFab: the design and construction of standard biological parts for metabolic engineering in *Saccharomyces cerevisiae*. Nucleic Acids Res. 43, e88.

Hamilton, S.R., Cook, W.J., Gomathinayagam, S., Burnina, I., Bukowski, J., Hopkins, D., Schwartz, S., Du, M., Sharkey, N.J., Bobrowicz, P., Wildt, S., Li, H., Stadheim, T.A., Nett, J.H., 2013. Production of sialylated O-linked glycans in *Pichia pastoris*. Glycobiology 23, 1192–1203.

Harju, S., Fedosyuk, H., Peterson, K.R., 2004. Rapid isolation of yeast genomic DNA: bust n' grab. BMC Biotechnol. 21, 8.

Heyland, J., Fu, J., Blank, L.M., Schmid, A., 2010. Quantitative physiology of *Pichia pastoris* during glucose-limited high-cell density fed-batch cultivation for recombinant protein production. Biotechnol. Bioeng. 107, 357–368.

Higuchi, R., Fockler, C., Dollinger, G., Watson, R., 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Nature 11, 1026–1030.

Inan, M., Fanders, S.A., Zhang, W., Hotez, P.J., Zhan, B., Meagher, M.M., 2007. Saturation of the secretory pathway by overexpression of a hookworm (*Necator americanus*) Protein (Na-ASP1). Methods Mol. Biol. 389, 65–76.

Invitrogen, 2002. Pichia Fermentation Process Guidelines. Invitrogen (Version B 053002).

Kaiser, C., Pototzki, T., Luttmann, R., Ellert, A., 2008. Applications of PAT-process analytical technology in recombinant protein processes with *Escherichia coli*. Eng. Life Sci. 8, 2.

Kelly, J.R., Rubin, A.J., Davis, J.H., Ajo-Franklin, C.M., Cumbers, J., Czar, M.J., Mora, K.d., Glieberman, A.L., Monie, D.D., Endy, D., 2009. Measuring the activity of BioBrick promoters using an in vivo reference standard. J. Biol. Eng. 3, 4.

Kubista, M., Andrade, J.M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., Ståhlberg, A., Zoric, N., 2006. The real-time polymerase chain reaction. Mol. Asp. Med. 27, 95–125.

Lantz, P.-G., Hahn-Hägerdal, B., Rådström, P., 1994. Sample preparation methods in PCR-based detection of food pathogens. Trends Food Sci. Technol. 5, 384–389.

Lee, C., Kim, J., Shin, S.G., Hwang, S., 2006. Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. J. Biotechnol. 123 (3), 273–280.

Lopes, A.G., Keshavarz-Moore, E., 2012. Prediction and verification of centrifugal dewatering of *P. pastoris* fermentation cultures using an ultra scale-down approach. Biotechnol. Bioeng. 109, 2039–2047.

Macauley-Patrick, S., Fazenda, M.L., McNeil, B., Harvey, L.M., 2005. Heterologous protein production using the *Pichia pastoris* expression system. Yeast 22, 4.

Mackay, I.M., 2004. Real-time PCR in the microbiology laboratory. Clin. Microbiol. Infect. 10, 190–212.

Malorny, B., Tassios, P.T., Rådström, P., Cook, N., Wagner, M., Hoorfar, J., 2003. Standardization of diagnostic PCR for the detection of foodborne pathogens. Int. J. Food Microbiol. 83, 39–48.

Miller, D.N., Bryant, J.E., Madsen, E.L., Ghiorse, W.C., 1999. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Appl. Environ. Microbiol. 65 (11), 4715–4724.

Müller, K.M., Arndt, K.M., 2012. Standardization in synthetic biology. Methods Mol. Biol. 813, 23–43.

Olson, N.D., Morrow, J.B., 2012. DNA extract characterization process for microbial detection methods development and validation. BMC. Res Notes 5, 668.

Pabinger, S., Rödiger, S., Kriegnera, A., Vierlingera, K., Weinhäusela, A., 2014. A survey of tools for the analysis of quantitative PCR (qPCR) data. Biomol. Detect. Quantif. 1, 23–33.

Parodi, B., Aresu, O., Bini, D., Lorenzini, R., Schena, F., Visconti, P., Cesaro, M., Ferrera, D., Andreotti, V., Ruzzon, T., 2002. Species identification and confirmation of human and animal cell lines: a PCR-based method. Biotechniques 32 (2), 432 (-4, 436, 438-40).

Plieskatt, J.L., Feng, Y., Rinaldi, G., Mulvenna, J.P., Bethony, J.M., Brindley, P.J., 2014. Circumventing qPCR inhibition to amplify miRNAs in plasma. Biomark Res. 22, 13.

Qiagen, 2006. Purification of Total DNA from Yeast Using the DNeasy Blood & Tissue Kit (DY13).

Queipo-Ortuño, M.I., Tena, F., Colmenero, J.D., Morata, P., 2008. Comparison of seven commercial DNA extraction kits for the recovery of Brucella DNA from spiked human serum samples using real-time PCR. Eur. J. Clin. Microbiol. Infect. Dis. 27 (2), 109–114.

Ruijter, J.M., Pfaffl, M.W., Zhao, S., Spiess, A.N., Boggy, G., Blom, J., Rutledge, R.G., Sisti, D., Lievens, A., De Preter, K., Derveaux, S., Hellemans, J., Vandesompele, J., 2013. Evaluation of qPCR curve analysis methods for reliable biomarker discovery: bias, resolution, precision, and implications. Methods 59, 32–46.

Rutledge, R.G., 2011. A java program for LRE-based real-time qPCR that enables large-scale absolute quantification. PLoS One 6, e17636.

Rutledge, R.G., Cote, C., 2003. Mathematics of quantitative kinetic PCR and the application of standard curves. Nucleic Acids Res. 31 (16), e93.
Rutledge, R.G., Stewart, D., 2008. A kinetic-based sigmoidal model for the polymerase chain reaction and its application to high-capacity absolute quantitative real-time PCR. BMC Biotechnol. 8, 47.
Rutledge, R.G., Stewart, D., 2010. Assessing the performance capabilities of LRE-based assays for absolute quantitative real-time PCR. PLoS One 5, e9731.
Sandén, A.M., Prytz, I., Tubulekas, I., Förberg, C., Le, H., Hektor, A., Neubauer, P., Pragai, Z., Harwood, C., Ward, A., Picon, A., De Mattos, J.T., Postma, P., Farewell, A., Nyström, T., Reeh, S., Pedersen, S., Larsson, G., 2003. Limiting factors in *Escherichia coli* fed-batch production of recombinant proteins. Biotechnol. Bioeng. 81, 158–166.
Schrader, C., Schielke, A., Ellerbroek, L., Johne, R., 2012. PCR inhibitors - occurrence, properties and removal. J. Appl. Microbiol. 113 (5), 1014–1026.
Shokere, L.A., Holden, M.J., Ronald Jenkins, G., 2009. Comparison of fluorometric and spectrophotometric DNA quantification for real-time quantitative PCR of degraded DNA. Food Control 20, 391–401.
Sovová, T., Křížová, B., Hodek, J., Ovesná, J., 2016. Identifying inhibitors/enhancers of quantitative real-time PCR in food samples using a newly developed synthetic plasmid. J. Sci. Food Agric. 96 (3), 997–1001.
Tolner, B., Smith, L., Begent, R.H.J., Chester, K.A., 2006. Production of recombinant protein in *Pichia pastoris* by fermentation. Nat. Protoc. 1, 1006–1021.
Tolvanen, K.E.S., Koskinen, P.E.P., Ylikoski, A.I., Ollikka, P.K., Hemmila, I.A., Puhakka, J.A., Karp, M.T., 2008. Quantitative monitoring of a hydrogen-producing *Clostridium butyricum* strain from a continuous-flow, mixed culture bioreactor employing real-time PCR. Int. J. Hydrog. Energy 33 (2), 542–549.
Yin, H., Liu, Z., Zhang, A., Zhang, T., Luo, J., Shen, J., Chen, L., Zhou, B., Fu, X., Fu, C., Zhang, Z., 2012. Intracellular expression and purification of the Canstatin-N protein in *Pichia pastoris*. Gene 504, 122–126.
Zhang, M., Wang, H.Y., 1994. Hydrogen peroxide production using chemically treated *Pichia pastoris* cells. Enzym. Microb. Technol. 16, 10–17.