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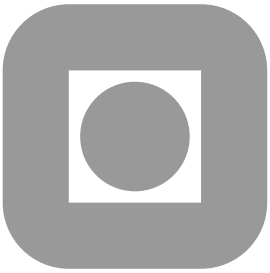

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A Bayesian hierarchical model for quantitative real-time PCR data

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Abstract

We present a Bayesian hierarchical model for quantitative real-time PCR data, aiming at relative quantification of DNA copy number in different biological samples. The model is specified in terms of a hidden Markov model for fluorescence intensities measured at successive cycles of the polymerase chain reaction. The efficiency of the reaction is assumed to depend on fluorescence intensities, and the relationship is specified based on the kinetics of the reaction. The model includes noise in the reaction process as well as measurement error. Taking a Bayesian inferential approach, marginal posterior distributions of the quantities of interest are estimated using Markov chain Monte Carlo. The method is applied to simulated data and an experimental data set.

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1 Introduction

Real-time quantitative PCR is a widely used technique for quantification of gene expression levels in a biological sample, in particular for low abundance genes. In contrast to microarray experiments, where the expression level of thousands of genes are measured simultaneously, real-time quantitative PCR is designed for targeted quantification of gene expression for a limited number of genes.

Starting from the biological sample, RNA is typically first isolated and reverse transcribed into complementary DNA. The initial target DNA is then amplified by the polymerase chain reaction (PCR). The PCR process consists of a series of repeated cycles, where at each cycle, a fraction of the target DNA is duplicated. The process is characterised by the efficiency, which, taking into account the intrinsic random nature of the process, can be defined as the probability that a molecule is duplicated at each cycle. In the initial phase of the reaction the efficiency is normally near one, while it decreases in the course of the reaction due to shortage of reaction material. Most commonly, the amount of target DNA at each cycle of the reaction is quantified by using fluorescence chemistry, and real-time PCR refers to continuous monitoring of the fluorescence intensities as the process proceeds. Examples of amplification curves, displaying the fluorescence intensities as a function of cycle, are shown in Section 4.2. For the first few cycles, the baseline cycles, the fluorescence intensities corresponding to the copy numbers of the target DNA are normally not distinguishable from background fluorescence. At the other end, as the efficiency decreases, the curve is eventually levelling out, reaching a plateau.

An underlying assumption in most quantitative PCR (qPCR) approaches, is that, disregarding the observational noise, the fluorescence intensity is proportional to the corresponding target DNA copy number. Consequently, whether or not the constant of proportionality is known, relative quantification of target DNA in two samples is possible, comparing the fluorescence intensities between the samples. However, in the presence of a series of baseline cycles, the fluorescence corresponding to the initial target DNA cannot be read directly from the amplification curve, and must be estimated from the curve.

Many currently available quantification methods are based on the assumption of a constant efficiency, implying exponential growth. In the approach by Livak and Schmittgen (2001), the efficiency is assumed to be equal to one, while in e.g. Gentle *et al.* (2001), Marino *et al.* (2003) and Cook *et al.* (2004), the assumption of exponential growth is utilised to estimate the efficiency from the amplification curve data. Strategies for identification of the cycles assumed to exhibit exponential growth have been suggested by e.g. Gentle *et al.* (2001), Liu and Saint (2002a) and Tichopad *et al.* (2003). Relaxing the constant efficiency assumption allows for the inclusion of data for a larger subset of the cycles. One approach that has been proposed is to fit parametric curves to the fluorescence intensities as a function of cycle number (Liu and Saint, 2002b; Rutledge, 2004), or to the sample efficiency (computed from the ratio of fluorescence for successive cycles) as a function of

intensity (Batsch *et al.*, 2008; Alvarez *et al.*, 2007). However, these are purely curve fitting methods, and do not model the PCR process as such.

Jagers and Klebaner (2003) propose to model the PCR as a branching process with an intensity dependent efficiency following the enzymological model for the PCR kinetics by Schnell and Mendoza (1997). The number of duplicates after each cycle is binomially distributed with probability given by the efficiency model. A modified version of the model is given by Lalam *et al.* (2004) and Lalam (2006), assuming that the efficiency is constant for the early cycles, but follows a damped version of the original efficiency model for intensities higher than a threshold value. The parameters of the efficiency model are estimated by conditional least squares, but no estimate of initial fluorescence for the target DNA is given.

In a relative qPCR experiment, the aim is to compare the gene expression levels of two or more conditions, and typically several replicates are made for each condition. We propose a Bayesian hierarchical model for relative qPCR, including observational noise, and estimating the model parameters based on data from all amplification curves jointly. With few exceptions, including Cook *et al.* (2004) and Batsch *et al.* (2008), this is in contrast to currently available methods for analysing qPCR data, which normally operate separately on each amplification curve. We quantify the uncertainty of the parameter estimates by their estimated posterior distributions, generated using Markov chain Monte Carlo methodology. Our approach is similar to that of Lalam (2007), but the latter is based on quantifying DNA from a single amplification curve, and on assuming constant efficiency. Our model is tested on a simulated data set, and results from running the algorithm on an experimental data set are also presented.

2 Model specification

We specify the model for a relative qPCR experiment, where the aim is quantitative comparison of the gene expression levels for two or more conditions, often a treatment and a control group. In what follows, we use the term treatment to refer to the experimental conditions to be compared. To control for non-biological effects that might influence the amplification process, the amplification curves for the gene of interest are usually contrasted with similar curves for a reference gene, that is expected not to be influenced by the treatments. We further consider the situation where the PCR is run in replicates for each treatment and gene combination. The model is specified for a qPCR experiment consisting of n amplification curves, representing replicated PCR runs for n_t treatments, and n_g genes for each treatment.

2.1 Full model

The model relies on the assumption that for each reaction i, each molecule has a probability $p_{i,k}$ to duplicate at cycle k, independently of the remaining molecules. Let $N_{i,k}$ denote the number of target DNA molecules for reaction i at cycle k. The stochastic model for the kinetics can be written

$$N_{i,k} = N_{i,k-1} + Z_{i,k}, \;\; i = 1, \ldots, n, \; k = 1, \ldots, m_i, \tag{1}$$

where

$$Z_{i,k} \mid N_{i,k-1}, p_{i,k} \sim \text{Binom}(N_{i,k-1}, p_{i,k}), \tag{2}$$

and m_i is the number of cycles for reaction i. For large $N_{i,k}$, the binomial distribution can be approximated by a normal distribution, such that

$$N_{i,k} \mid N_{i,k-1}, p_{i,k} \sim \mathcal{N}(N_{i,k-1}(1 + p_{i,k}), N_{i,k-1}p_{i,k}(1 - p_{i,k})), \;\; i = 1, \ldots, n, \; k = 1, \ldots, m_i. \tag{3}$$

The number of molecules after each cycle is measured in terms of the corresponding fluorescence intensity. We adopt the commonly made assumption that the fluorescence is proportional to the number of molecules, but will assume in addition that the fluorescence is measured with additive noise. Let $x_{i,k} = \gamma N_{i,k}$, such that $x_{i,k}$ represents noise-free fluorescence. Conditionally on the reaction history, the variable $x_{i,k}$ will then also be approximately normal, with mean and variance given by

$$\begin{aligned} \text{E}(x_{i,k} \mid N_{i,k-1}, p_{i,k}) &= \gamma N_{i,k-1}(1 + p_{i,k}) & (4) \\ \text{Var}(x_{i,k} \mid N_{i,k-1}, p_{i,k}) &= \gamma^2 N_{i,k-1} p_{i,k}(1 - p_{i,k}). & (5) \end{aligned}$$

Substituting $x_{i,k-1}/\gamma$ for $N_{i,k-1}$, we get the model

$$x_{i,k} \mid x_{i,k-1}, p_{i,k}, \gamma \sim \mathcal{N}(x_{i,k-1}(1 + p_{i,k}), \gamma x_{i,k-1} p_{i,k}(1 - p_{i,k})), \;\; i = 1, \ldots, n, \; k = 1, \ldots, m_i \tag{6}$$

for $x_{i,k}$. We further assume that the initial fluorescence $x_{i,0}$ for curve i is normally distributed with gene and treatment dependent means μ_{g_i,t_i}, and with variance $\kappa_{x0}^{-1}\mu_{g_i,t_i}$ proportional to the mean. Here, g_i and t_i denote the considered gene (g) and treatment (t) corresponding to amplification curve i. That is,

$$x_{i,0} \mid \mu_{g_i,t_i}, \kappa_{x_0} \sim \mathcal{N}(\mu_{g_i,t_i}, \kappa_{x_0}^{-1}\mu_{g_i,t_i}), \;\; i = 1, \ldots, n. \tag{7}$$

Our model for the efficiency $p_{i,k}$ of the reaction is motivated by the enzymological model for the PCR kinetics presented in Schnell and Mendoza (1997). Following their model, the efficiency $p_{i,k}$, going from cycle $k-1$ to k, and with $N_{i,k-1}$ molecules at cycle $k-1$, is

$$p_{i,k} = \frac{K_{g_i,t_i}}{K_{g_i,t_i} + N_{i,k-1}}, \tag{8}$$

where K_{g_i,t_i} is the Michaelis-Menten reaction constant, assumed to depend on gene and treatment. We restrict our model to the subset of cycles for which (8) can be assumed to be a reasonable description of the process. Substituting $x_{i,k-1}/\gamma$ for $N_{i,k-1}$, we get

$$\begin{aligned} \text{logit}(p_{i,k}) &= \log\left(\frac{p_{i,k}}{1-p_{i,k}}\right) \quad (9) \\ &= \log\left(\frac{\gamma K_{g_i,t_i}}{\gamma K_{g_i,t_i}+x_{i,k-1}}/(1-\frac{\gamma K_{g_i,t_i}}{\gamma K_{g_i,t_i}+x_{i,k-1}})\right) \\ &= \log(\gamma K_{g_i,t_i}) - \log(x_{i,k-1}). \quad (10) \end{aligned}$$

We consider $\alpha_{g_i,t_i} = \log(\gamma K_{g_i,t_i})$ as unknown constants to be estimated from the observed amplification curve. In addition, we allow the coefficient of the $\log(x_{i,k-1})$ term to be different from -1, and adding normally distributed noise, we arrive at the following model for the efficiency $p_{i,k}$:

$$\text{logit}(p_{i,k}) \mid x_{i,k-1}, \alpha_{g_i,t_i}, \beta_{g_i,t_i}, \tau_p \sim \mathcal{N}(\alpha_{g_i,t_i} + \beta_{g_i,t_i}\log(x_{i,k-1}), \tau_p^{-1}),\ \forall i,k. \quad (11)$$

As before, the indices $g = g_i$ and $t = t_i$ are used to represent the gene and treatment corresponding to curve i, respectively.

Finally, we assume that fluorescence intensity is observed with additive normally distributed noise, and that the distribution for the observed fluorescence $y_{i,k}$ for cycle k of curve i is given by

$$y_{i,k} \mid x_{i,k}, \tau_y \sim \mathcal{N}(x_{i,k}, \tau_y^{-1}),\ \ i = 1,\ldots,n,\ k = 1,\ldots,m_i. \quad (12)$$

In (12) we have implicitly assumed that $y_{i,k}$ represents background corrected data. In principle, background correction could be included in the model and estimated jointly with the remaining parameters, but we will assume that the amplification curves are background corrected in a preprocessing stage. The full hierarchical model is illustrated in Figure 1.

In a typical experiment $n_g = 2$ and $n_t = 2$, and the main parameter of interest is the ratio of the mean initial abundance for a treated and a control group for the gene of interest, adjusted for the corresponding ratio for a reference gene. In terms of the model parameters, this ratio, which we denote by R_{adj}, is

$$R_{\text{adj}} = \frac{\mu_{2,2}/\mu_{2,1}}{\mu_{1,2}/\mu_{1,1}}, \quad (13)$$

where the first subscript (1 or 2) denotes the reference gene and the gene of interest, respectively, and the second subscript (1 or 2) denotes the control and treated groups.

2.2 Simplified model

Observe that in the full model, stochastic growth is represented both by the model (11) for the efficiencies $p_{i,k}$, and in the normal model (6) for the fluorescence intensities, conditionally on $p_{i,k}$. Fitting the full model using the Markov chain Monte Carlo (MCMC)

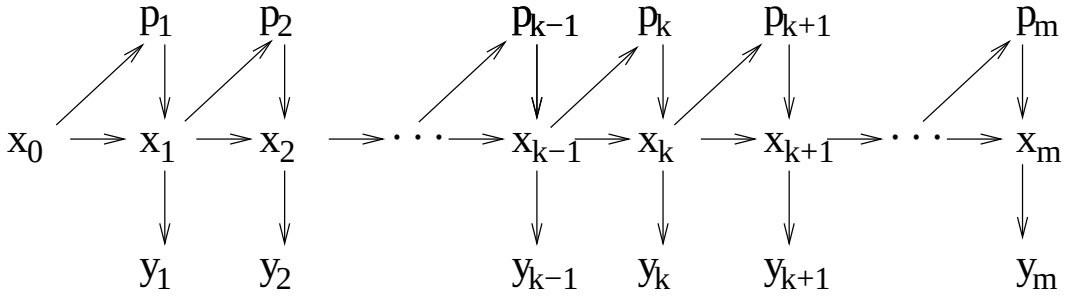

Figure 1: *Graphical description of the full hierarchical model for a single reaction. Here, y, x and p are used to denote the observed fluorescence intensities, the noise-free intensities, and the efficiency, respectively. The reaction subscript i is suppressed, and the cycle independent parameters of the model are not shown.*

approach described in Section 3 to simulated data sets, we experienced that mixing and convergence of the MCMC algorithm were slow. Using a single-site MCMC algorithm, this is not surprising, in light of the correlation structure inherent in the model. Further, some of the parameters of the full model of Section 2.1 appears to be hard to identify from the data. This applies in particular to the scaling factor γ in (6), representing the link between the fluorescence intensity and the number of molecules.

We therefore propose a simplified version of the full model, ignoring the noise in the model for the intensities $x_{i,k}$ as specified in (6). It turns out that the resulting model gives reasonable mixing and convergence for the corresponding MCMC algorithm. An alternative simplification is to ignore the error in the efficiency model (11), but this does not lead to a similar improvement. The chosen simplification has the advantage that the scaling factor γ is eliminated from the model. Also, attempts to quantify this scaling factor from amplification data (see e.g. Rutledge (2004) and Goll *et al.* (2006)), indicate that the factor is small relative to typical fluorescence intensities. Consequently, the conditional variance for $x_{i,k}$ is expected to be relatively small, further motivating the proposed simplification.

By substituting $x_{i,k}$ by the conditional mean $x_{i,k-1}(1+p_{i,k})$ in the likelihood (12) and in the model (11) for the efficiency, the simplified model can be summarised by

$$y_{i,k} \mid x_{i,0}, p_{i,1}, \ldots, p_{i,k}, \tau_y \sim \mathcal{N}\left(x_{i,0}\prod_{j=1}^{k}(1+p_{i,j}), \tau_y^{-1}\right), \quad (14)$$

$$\begin{aligned}\operatorname{logit}(p_{i,k}) \mid \alpha_{g_i,t_i}, \beta_{g_i,t_i}, x_{i,0}, p_{i,1}, \ldots, p_{i,k-1}, \tau_p \sim \\ \mathcal{N}\left(\alpha_{g_i,t_i} + \beta_{g_i,t_i}\log\left(x_{i,0}\prod_{j=1}^{k-1}(1+p_{i,j})\right), \tau_p^{-1}\right),\end{aligned} \quad (15)$$

$$x_{i,0}|\mu_{g_i,t_i}, \kappa_{x_0} \sim \mathcal{N}\left(\mu_{g_i,t_i}, \kappa_{x_0}^{-1}\mu_{g_i,t_i}\right), \quad (16)$$

for $i = 1, \ldots, n$ and $k = 1, \ldots, m_i$.

3 Parameter estimation

We take a Bayesian approach, and estimate marginal posterior distributions for the unknown quantities of the model by Markov chain Monte Carlo (MCMC). The model specification is based on the assumption that a valid range of cycles corresponding to the chosen model for $p_{i,k}$ given in (15) has been identified. We first describe how the valid cycle window is selected for an experimental data set, and then describe the sampling algorithm.

3.1 Selection of the cycle window

In the course of a PCR run, reaction conditions will change. Due to shortage of reaction materials, the amplification curve will eventually level out, meaning that the efficiency approaches zero. The limiting mean growth rate corresponding to the model given in (15) is linear growth, implying that not all data points of an amplification curve can be included in the analysis. We select the valid cycle window by identifying the inflection point of the amplification curve from a four-parameter sigmoid curve fitted to the data, and choose the closest cycle prior to this inflection point as the final cycle m_i for each curve i. In addition, we have observed that even after background correction, there are trends for the early cycles that cannot be explained by the amplification process. The data for these cycles are discarded from the analysis. The cycles to be ignored are selected using the method in Tichopad *et al.* (2003) for identifying the final baseline cycle, and then discarding data for the cycles prior to and including this cycle. Taking this approach, we will normally discard more data than necessary, and selecting a maximal valid cycle window represents a topic for further study.

3.2 The MCMC algorithm

To complete the model specification, we assign prior distributions to the cycle independent parameters of the model. Let $\boldsymbol{\theta} = (\tau_y, \tau_p, \kappa_{x_0}, \{\mu_{g,t}\}, \{\alpha_{g,t}\}, \{\beta_{g,t}\})^T$ be the vector of these parameters. Their priors are summarised in Table 1. The mean and precision of the normal priors for $\alpha_{g,t}$ and $\beta_{g,t}$, and the precision of the truncated normal prior for $\mu_{g,t}$, are computed by first specifying what we believe is a reasonable range for each of the these parameters, and then selecting the parameters of the distribution such that a prior probability of approximately 0.95 is assigned to that range. The precision parameters are all assigned non-informative priors.

The unknown quantities to be estimated are the vector of efficiencies $\boldsymbol{p}_i,\ i = 1, \ldots, n$ for cycles 1 to m_i for each curve i, the initial fluorescence, $x_{i,0},\ i = 1, \ldots, n$, and the parameter vector $\boldsymbol{\theta}$. The vector of fluorescence intensities $\boldsymbol{x}_i = (x_{i,1}, \ldots, x_{i,m_i})$ for each amplification curve can be computed from $x_{i,0}$ and $\boldsymbol{p}_i$ by $x_{i,k} = x_{i,0} \prod_{j=1}^{k}(1 + p_{i,j})$. We let $\boldsymbol{y}_i$ denote the

$\mu_{g,t} \sim \mathcal{N}(0, 5.0^2) I(\mu_{g,t} > 0),\ g = 1, \ldots, n_g,\ t = 1, \ldots, n_t$
$\kappa_{x_0} \sim \text{Gamma}(0.1, 0.00001)$
$\alpha_{g,t} \sim \mathcal{N}(10.0, 7.5^2),\ g = 1, \ldots, n_g,\ t = 1, \ldots, n_t$
$\beta_{g,t} \sim \mathcal{N}(-1.5, 0.5^2),\ g = 1, \ldots, n_g,\ t = 1, \ldots, n_t$
$\tau_p \sim \text{Gamma}(0.5, 0.0005)$
$\tau_y \sim \text{Gamma}(0.5, 0.0005)$

Table 1: *Summary of the prior distributions for the cycle independent parameters. Here, n_g and n_t denotes the number of genes and treatments, respectively.*

vector of corresponding observed fluorescence for reaction i.

The MCMC algorithm works by sampling from a Markov chain with the distribution of interest as stationary distribution, in our problem the joint posterior distribution. Except for the parameters $\alpha_{g,t}$ and $\beta_{g,t}$, we sample each parameter at a time from the full conditional distribution given the remaining parameters. If the full conditional distribution is non-standard, we apply a Metropolis-Hastings step. We then first generate a value from a proposal distribution given the current sample, and then accept or reject this value with a probability depending on the full conditional distribution and the proposal distribution.

The full conditional distributions of the precisions τ_p and τ_y and the parameter κ_{x_0} are Gamma distributions, and these full conditional distributions can be sampled from directly. The two parameters $\alpha_{g,t}$ and $\beta_{g,t}$ for each pair (g, t) are sampled jointly from their binormal full conditional distribution.

For the parameters $\mu_{g,t}$, as well as for the efficiencies $p_{i,k}$ and the initial fluorescence $x_{i,0}$, the full conditional distributions are non-standard, and we apply a Metropolis-Hastings step for each of these parameters. We use normal proposal distributions centred at the current sample. The standard deviations of the proposal distributions are tuned by repeatedly running a series of 5000 samples, and adjusting the standard deviations such that the acceptance probabilities are approximately between 0.2 and 0.5.

The sampling routine is implemented in C.

4 Results

To illustrate our approach, we present results from applying it to a simulated and an experimental data set.

4.1 Results for a simulated data set

To assess the performance of the model and the MCMC algorithm, we run the algorithm on a data set simulated from the model. We simulate a set of data with $n = 12$ samples, representing three replicates of each pair of $n_g = 2$ genes and $n_t = 2$ treatments, using the parameter values given in Table 2. The values for $\alpha_{g,t}$ are calculated as $\alpha_{g,t} = \text{logit}(0.999999) - \beta_{g,t}\log(\mu_{g,t})$, using the selected values for $\beta_{g,t}$ and $\mu_{g,t}$. The total number of cycles for the twelve curves are $\boldsymbol{m} = (17, 17, 17, 18, 18, 18, 20, 20, 20, 20, 20, 20)$, and the simulated amplification curves are shown in Figure 2.

$\mu_{1,1} = 0.2$	$\alpha_{1,1} = 12.206$
$\mu_{1,2} = 0.1$	$\alpha_{1,2} = 11.513$
$\mu_{2,1} = 0.025$	$\alpha_{2,1} = 10.127$
$\mu_{2,2} = 0.025$	$\alpha_{2,1} = 10.127$
$\kappa_{x_0} = 1000$	$\beta_{g,t} = -1.0,\ \forall(g,t)$
$\tau_y = 1/40^2$	$\tau_p = 64.0$
$R_{adj} = \dfrac{\mu_{2,2}/\mu_{2,1}}{\mu_{1,2}/\mu_{1,1}} = 0.5$	

Table 2: *Model parameters for the simulated data set.*

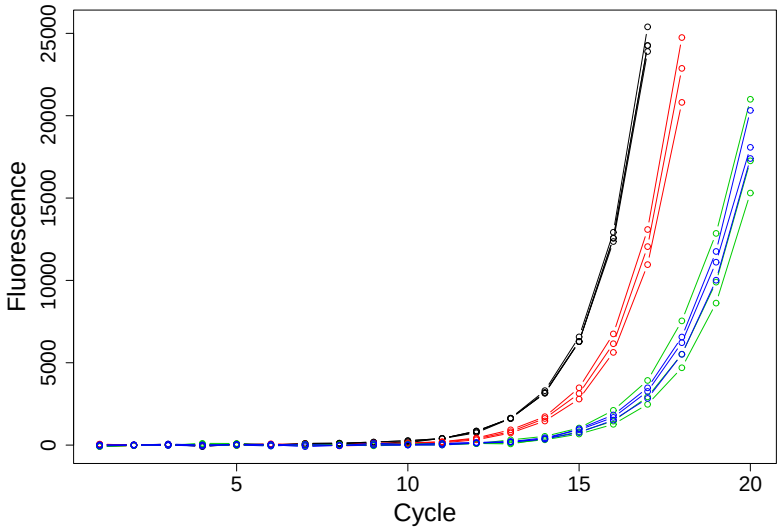

Figure 2: *Amplification curves for the simulated data set. Each colour represents triplicates of a gene and treatment combination* (g, t)*: Black* $= (1, 1)$*, red* $= (1, 2)$*, green* $= (2, 1)$*, and blue* $= (2, 2)$*.*

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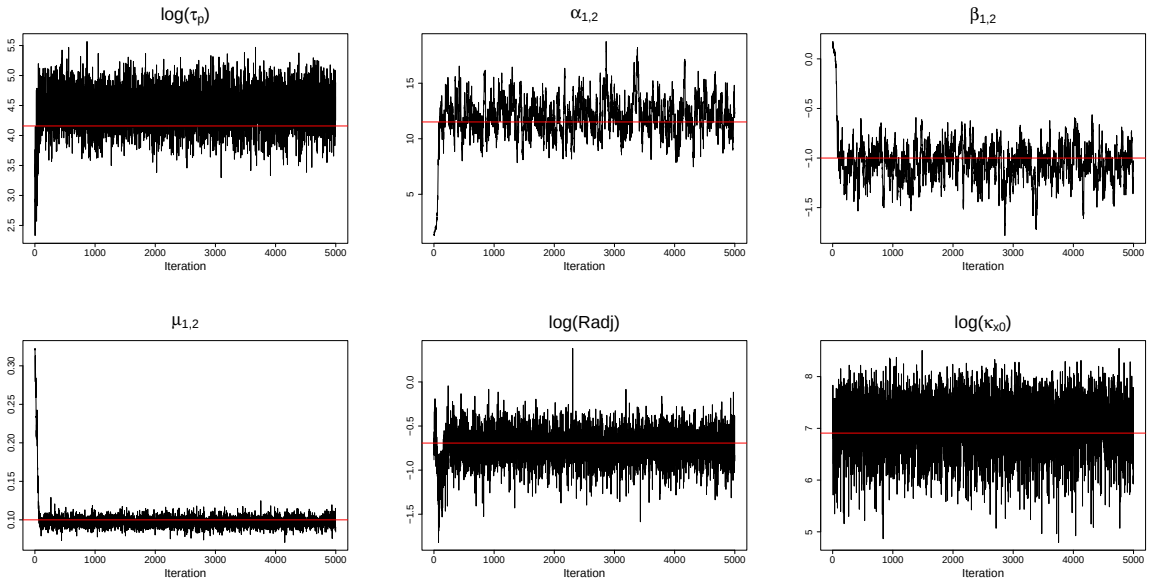

Figure 3: *Trace plots for some of the parameters for the simulated data set. Every 4000th iteration is shown. The horizontal lines indicate the true values.*

The algorithm was run for 20 mill. iterations, taking 4h 17min of CPU time on a 2.66 GHz Unix system. In Figure 3 trace plots of samples from the MCMC algorithm are shown for a subset of the parameters. We discard the first 500 thinned iterations as burn-in iterations. Estimated posterior means and 95% credibility intervals for the parameters were computed from the remaining 4500 iterations, and the results are given in Table 3, together with the true values. For the efficiency parameters, posterior means and 95% credibility intervals are shown in Figure 4 for three reactions. We observe that the parameter values used to generate the data set are well reproduced.

4.2 Results for an experimental data set

The model was fitted to an experimental data set comparing gene expression in rats treated with Octreotide long-acting release (LAR) to untreated controls. The gene of interest, KLF4, was contrasted with the reference gene β-actin, and the amplifications were run in triplicates for each of the four treatment and gene combinations, in total $n = 12$ reactions. The experimental protocol of the data set is described in Appendix A. The aim of the study was not a thorough analysis of the specific data set, but to investigate the applicability of the method to experimental data.

The data were background corrected by first manually selecting a window of 6-7 observations that were apparent baseline cycles, and subtracting the average fluorescence of these cycles from all observations. The twelve background corrected amplification curves are

Parameter	Posterior mean	95% credibility interval		True value
		Lower limit	Upper limit	
τ_p	91.7	44.9	160	64
$\alpha_{1,1}$	14.5	9.76	19.9	12.2
$\beta_{1,1}$	-1.24	-1.81	-0.726	-1
$\alpha_{1,2}$	12.1	9.09	15.4	11.5
$\beta_{1,2}$	-1.06	-1.42	-0.729	-1
$\alpha_{2,1}$	10.4	8.87	12.1	10.1
$\beta_{2,1}$	-1.04	-1.23	-0.863	-1
$\alpha_{2,2}$	10.3	8.66	11.9	10.1
$\beta_{2,2}$	-1.01	-1.2	-0.832	-1
τ_y	0.000578	0.000465	0.000699	0.000625
κ_{x_0}	1310	374	2890	1000
$\mu_{1,1}$	0.197	0.181	0.213	0.2
$\mu_{1,2}$	0.0974	0.0863	0.109	0.1
$\mu_{2,1}$	0.0259	0.0204	0.0321	0.025
$\mu_{2,2}$	0.0264	0.021	0.0328	0.025
R_{adj}	0.491	0.34	0.68	0.5

Table 3: *Estimated posterior means and 95% credibility intervals for the model parameters of the simulated data set.*

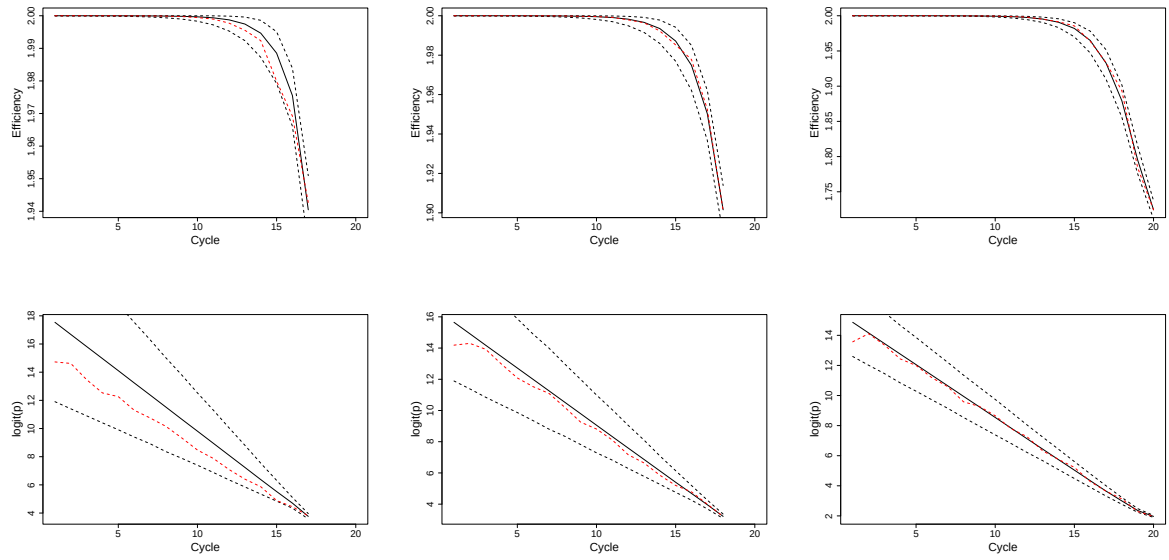

Figure 4: *Estimated posterior means (full lines) and 95% credibility intervals (black dashed lines) for the efficiency p (upper panels) and logit(p) (lower panels) for three of the reactions. The realisations of p for the simulated data set are added as red dashed lines.*

shown in the left panel of Figure 5. For each of the amplification curves, the approach described in Section 3.1 was used to select the cycle window corresponding to the efficiency model (15). The selected cycle windows for the twelve amplification curves are illustrated in the right panel of Figur 5.

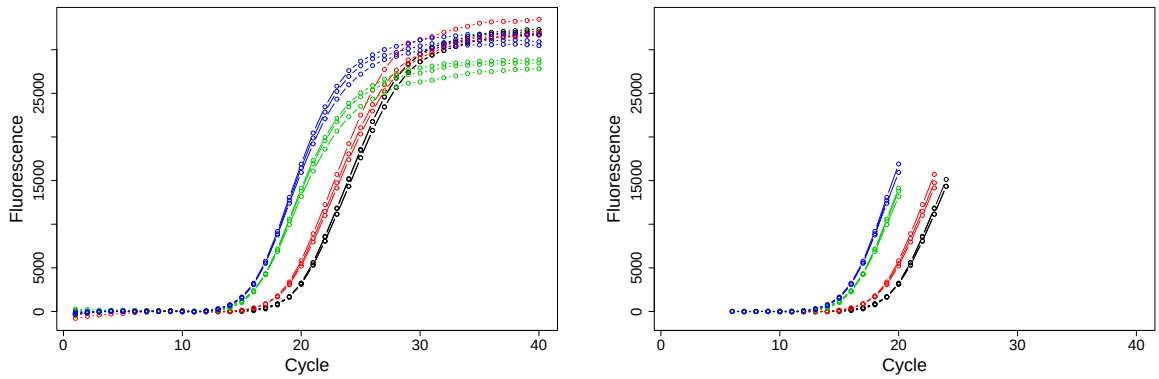

Figure 5: *Amplification curves for treated (red: KLF4, blue: reference gene) and untreated (black: KLF4, green: reference gene) rats for the Octreotide LAR data set. The right panel shows the cycle windows used in the analysis.*

The simplified model was fitted using the MCMC algorithm described in Section 3, running the algorithm for 50 mill. iterations. The convergence of the algorithm was monitored by visual inspection of trace plots. The mixing was found to be reasonable, and the algorithm was considered to have converged. Resulting trace plots for a few model parameters are shown in Figure 3. After thinning to every 10000th iteration to reduce autocorrelation for successive iterations, the initial 1000 iterations are considered burn-in iterations, leaving 4000 iterations for further analysis.

Estimated posterior means and 95% credibility intervals for the model parameters are listed in Table 4. Similar results for the initial fluorescence for the individual amplification curves, $x_{i,0},\ i = 1, \ldots, n$, and for the efficiencies $p_{i,k}$, for a selection of the curves, are illustrated in Figures 7 and 8. The efficiencies are estimated to decrease from nearly 1 to between 0.27 and 0.42 for the twelve curves. The precision τ_p of the efficiency model (15) is estimated to be relatively high, and the posterior variability on logit-scale decreases with cycle. The latter seems reasonable since, relative to the fluorescence intensities, the noise of the amplification curves is expected to be largest close to the baseline cycles. From Figure 7 we observe that the credibility intervals for the gene and treatment dependent means of the initial fluorescence are of similar width as the corresponding intervals for the individual initial values. This might seem counter-intuitive, but we should keep in mind that the estimates are based on only three replicates within each gene and treatment group.

The estimated marginal posterior density of the main parameter of interest, the ratio R_{adj} (13), is shown in the right panel of Figure 7. The gene of interest, KLF4, is estimated

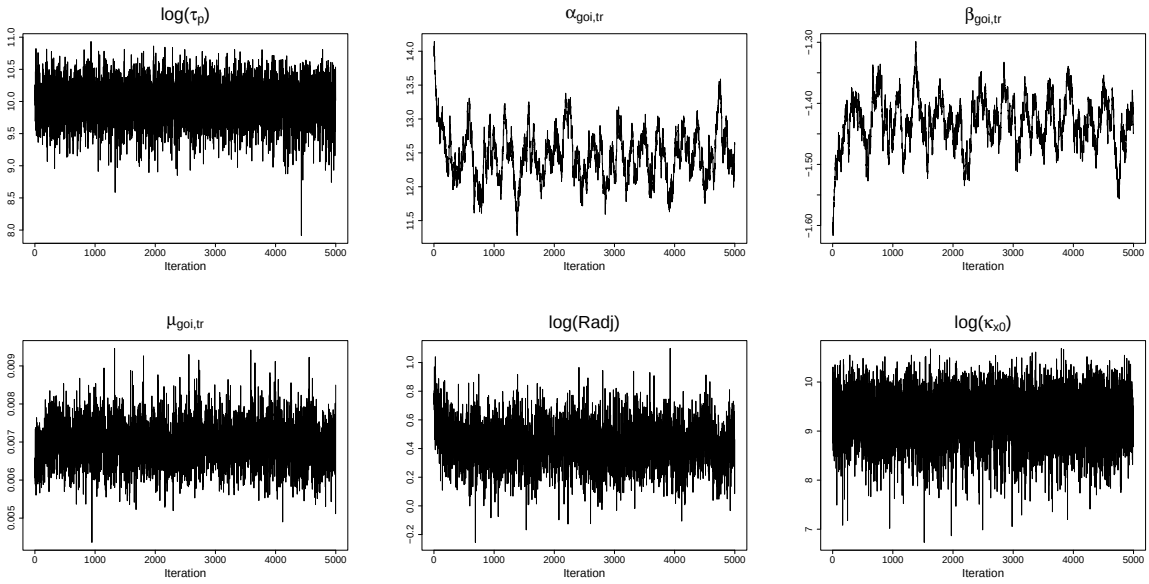

Figure 6: *Trace plots for some of the parameters for the Octreotide LAR data set. Every 10000th of 50 mill. iterations are shown.*

to be up-regulated in the treated group compared to the control group. The posterior mean gives a point estimate of 1.53, and using the sample quantiles, we arrive at a 95% credibility interval between 1.15 and 1.99. The estimated posterior probability that R_{adj} exceeds 1 is $P(R_{adj} > 1 \mid \boldsymbol{y}_1, \ldots, \boldsymbol{y}_n) = 0.996$. This means that the posterior probability that the gene of interest is up-regulated in the treated rats is estimated to 0.996.

5 Discussion

We have presented a Bayesian hierarchical model for quantitative real-time PCR data, based on a generalisation of the branching process model of Jagers and Klebaner (2003). The model allows for fluorescence intensity dependent efficiency, and includes noise in the model for the reaction process as well as measurement error.

The approach relies on the assumption that the model for the efficiency is valid in the selected cycle window, but it is not restricted to the specific model given in (15). In principle, any model describing the relationship between efficiency and fluorescence intensity or cycle can be used, as long as a corresponding cycle window representing the valid cycles for the model can be identified.

In the full model in Section 2.1, we motivate the model for the fluorescence intensities by a normal approximation to a binomial distribution of the number of new copies at each cycle. This assumption can be questioned for small copy numbers combined with large

Parameter	Posterior mean	95% credibility interval	
		Lower limit	Upper limit
τ_p	22900	11200	38800
$\alpha_{\text{goi,ctrl}}$	13	11.9	14.1
$\beta_{\text{goi,ctrl}}$	-1.49	-1.61	-1.36
$\alpha_{\text{goi,tr}}$	12.4	11.8	13.2
$\beta_{\text{goi,tr}}$	-1.43	-1.51	-1.36
$\alpha_{\text{ref,ctrl}}$	13.9	12.6	15.3
$\beta_{\text{ref,ctrl}}$	-1.59	-1.74	-1.44
$\alpha_{\text{ref,tr}}$	14.7	13.8	15.9
$\beta_{\text{ref,tr}}$	-1.65	-1.78	-1.55
τ_y	9.91e-05	7.81e-05	0.000122
κ_{x_0}	12300	3260	27600
$\mu_{\text{goi,ctr}}$	0.00336	0.0027	0.00412
$\mu_{\text{goi,tr}}$	0.00694	0.00592	0.00803
$\mu_{\text{ref,ctrl}}$	0.0366	0.0338	0.0396
$\mu_{\text{ref,tr}}$	0.0499	0.0467	0.0533
R_{adj}	1.54	1.15	2.04

Table 4: *Estimated posterior means and 95% credibility intervals for the model parameters for the Octreotide LAR data set. Here, 'goi' and 'ref' denote the gene of interest and the reference gene, respectively, and 'tr' and 'ctrl' the treated and control groups.*

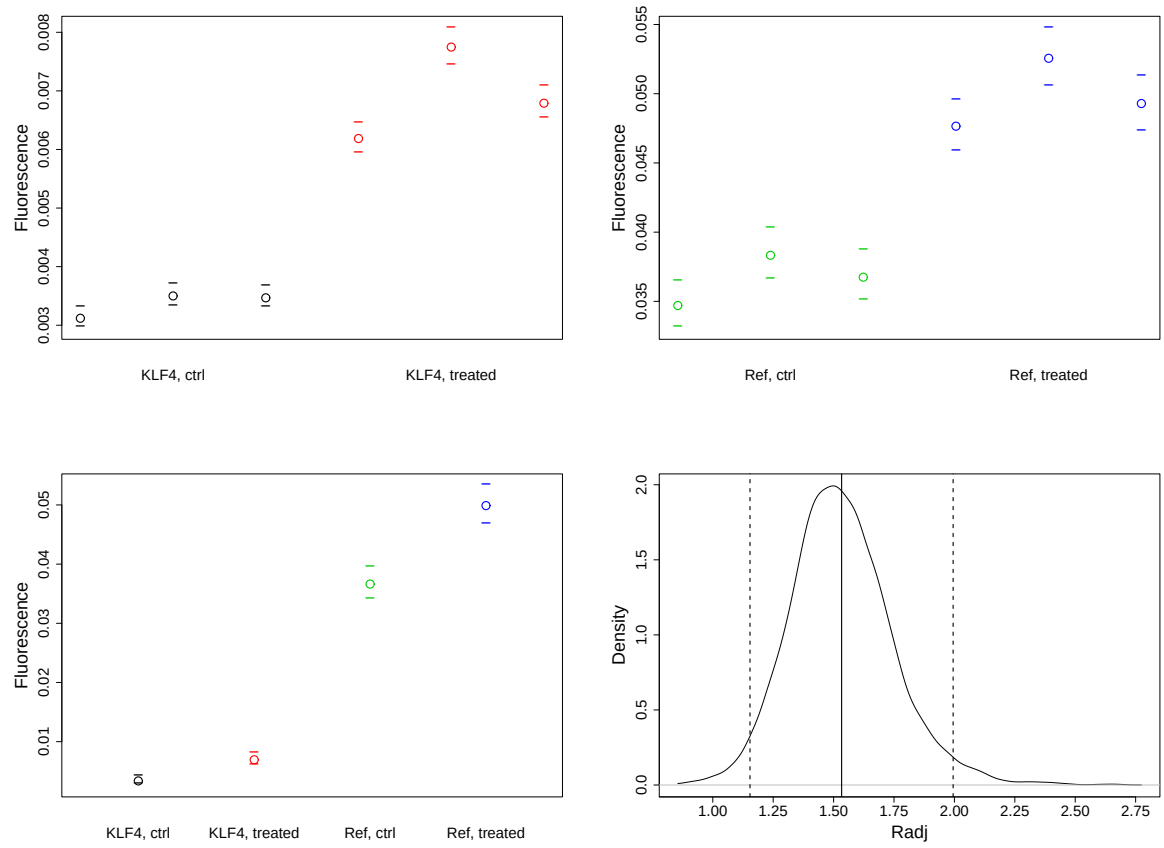

Figure 7: *Estimated posterior means and 95% credibility intervals for the initial fluorescence, $x_{i,0}$ (top panels), for the mean initial fluorescence, $\mu_{t,g}$ (bottom left panel), and for R_{adj} (bottom right panel). The vertical lines indicate the posterior mean and 95% credibility interval for R_{adj}. Note the differences in range for the y-axes for the two top panels.*

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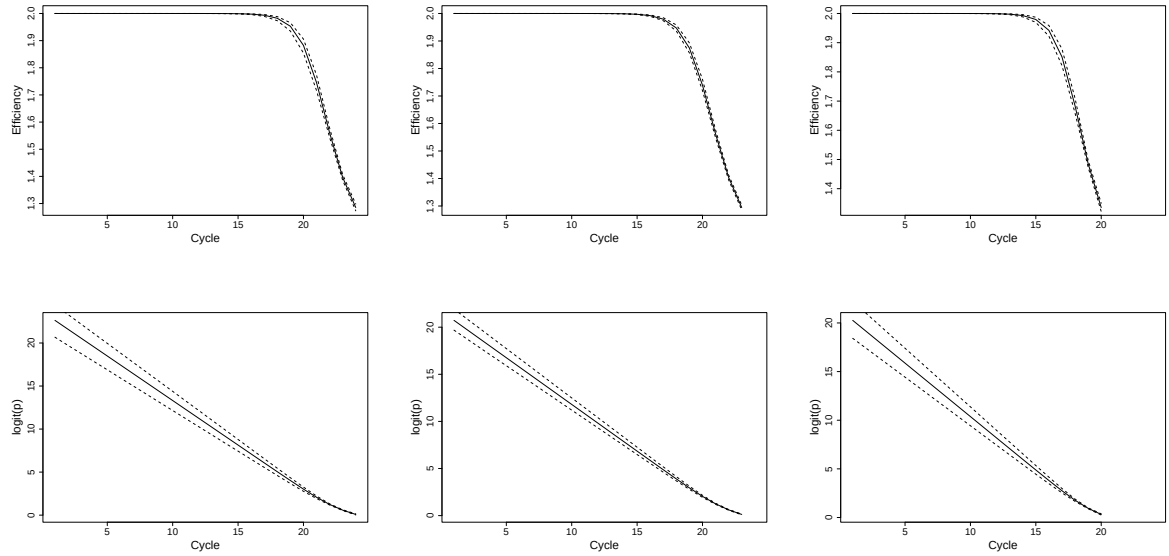

Figure 8: *Estimated posterior means (full lines) and 95% credibility intervals (black dashed lines) for the efficiency p (upper panels) and logit(p) (lower panels) for three of the reactions for the Octreotide LAR data set.*

efficiencies. However, in the simplified model in Section 2.2 the noise in (6) is ignored, and no such assumption is needed.

Due to the Markov structure of the model, the noise-free fluorescence $\boldsymbol{x}$ and the efficiencies $\boldsymbol{p}$ are highly auto- and cross-correlated in the full model. In the simplified model, auto-correlation for the efficiencies is still a source of slow mixing in the single-site MCMC algorithm. Block sampling algorithms are known to improve mixing and convergence properties, and blocking $\boldsymbol{x}$ and $\boldsymbol{p}$, as well as related hyperparameters, could be explored. However, a challenge of such an approach is to find joint proposal distributions that give reasonable acceptance probabilities.

The model is based on the assumption that the amplification curve data are background corrected in a preprocessing step, and the choice of background correction have potentially a strong impact on the results. In principle, background correction could be included in the model by adding a linear or non-linear term to the mean of the likelihood (14). However, parameter identifiability might become an issue in this case, and introducing background correction into the model remains a topic for further study.

We have focused on relative quantification, estimating the ratio R_{adj} (13). However, if an estimate of the scaling factor γ, relating fluorescence to DNA copy number, is available, the approach can in principle also be used to quantify the absolute amount of target DNA in a biological sample from the estimated initial fluorescence.

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A Experimental protocol for the Octreotide LAR data

The animal experiments were approved by the Animal Welfare Committee of St.Olav's University Hospital. Two groups of female Sprague-Dawley rats (body weight 193-227 g) were used, one group received Octreotide LAR and a control group received the LAR vehicle. After 21 days the rats were anaesthesized, drained for blood and gastric oxyntic mucosa was isolated. (For a full description of the experimental procedure see Erlandsen *et al.* (2007)). Total RNA for qPCR was isolated using RNeasy Midi Kit (Qiagen, Valencia, CA). cDNA synthesis and qPCR were performed using iScript^{TM}cDNA Synthesis Kit and iQ^{TM} SYBR$^{®}$ Green Supermix (Bio-Rad Laboratories, Hercules, CA), respectively, and the qPCR reactions were done on the Mx3000P^{TM}Real-Time PCR System (Stratagene, La Jolla, CA). The gene of interest and reference gene used in the experiment were the KLF4 (Unigene-ID Rn. 7719) and β-actin (Unigene-ID Rn. 94978). The gene specific primers used for KLF4 were; forward primer: 5'-CTTGTGACTATGCAGGCTGT-3', reverse primer: 5'-AGTGCCTGGTCAGTTCATCT-3'. The primers for the reference gene were; forward primer: 5'-CTGGCTCCTAGCACCATGA-3', reverse primer: 5'-AGCCACCAATCCACACAGA-3'. The PCR temperature profile was: once for 2 min at 95 °C (activation), then 40 reaction cycles of 20 sec at 95 °C (denaturation), 30 sec (optimised annealing temperature), 40 sec at 72 °C (synthesis) and finally 5 min at 72 °C (elongation) and meltingpoint determination.