Sequencing Error Adjusted and Multiple Comparison Corrected Read Depth Estimation

Introduction
A critical component in planning Next Generation Sequencing (NGS) experiments is determining appropriate read depth. This is particularly important for reliably detecting what if any differences exist between two biological samples, for example normal versus tumor tissue. The problem of determining appropriate read depth can be thought of as a sample size estimation problem with two important considerations. The first is the inclusion of systematic sequencing errors (SSE) on read accuracy; this can include quality score and other metrics. The second is controlling for multiple comparisons, which in the context of NGS is especially important as we are comparing millions/billions of locations between the two samples.

In this analysis we develop a Bayesian method for helping to determine read depth and associated power estimates, taking into account both sequencing error and false discovery rate. Our specific focus will be to provide read depth estimates in the context of sample homogeneity assessment; i.e. looking at two sequences grown from the same batch and determining how deeply those sequences need to be sequenced in order to detect whether any differences exist.

Sampling Distribution
As this type of analysis is done prospectively to help determine appropriate read depth coverage for the final sample preparations, we work with simulated data only as no measurements have been taken. In order to arrive at an estimate of read depth we need a way to simulate observed purities, i.e. adjusted for SSEs and other distributional factors associated with an NGS experiment. Our objective is to compare the purities, simulated from two vials and determine if differences could be detected as we slowly change the underlying true purity of one vial, while holding the other fixed. Stated in terms of a hypothesis test we have

\[ H_0: \text{purity of vial}_1 = \text{purity of vial}_2 \text{ vs. } H_A: \text{vial purities not equal}. \]

To generate these purities we used a Bayesian sampling scheme as a first step in our analysis, this is done prior to any adjustment for multiple comparisons which we describe in the following section. We now describe the sampling procedure.

We begin by defining the terms of our model (note that these all pertain to the sampling of a purity associated with a base at a given position in the genome for a single vial), letting denote \( r \) read depth we have

\[ B_i = \text{The event that the reference base } b \in \{A, C, T, G\} \text{ is matched in read } i, i = 1, \ldots, r, \]
\[ E_i = \text{The occurrence of a sequencing error at read } i, i = 1, \ldots, r, \]
For each of these we assume the following probability distributions:

\[
\begin{align*}
(B_i|E_i = 0, p_b) & \sim \text{Bernoulli}(p_b), \ i = 1, \ldots, r \\
(B_i|E_i = 1, p_b) & \sim \text{Bernoulli}(1 - p_b), \ i = 1, \ldots, r \\
E_i & \sim \text{Bernoulli}(\epsilon), \ i = 1, \ldots, r \\
\Pr(p_b) & = \text{Beta}(\alpha, \beta)
\end{align*}
\]

The conditional distributions of the data can be understood as follows. For the case given by \((B_i|E_i = 0, p_b)\), i.e. where no sequencing errors have occurred, the probability of observing the correct reference base is related to the purity of that base, \(p_b\) and follows a \(\text{Bernoulli}(p_b)\) distribution. For the case where a sequencing error has occurred, \((B_i|E_i = 1, p_b)\) the conditional distribution captures the event that the incorrect base is sampled but is identified as the reference base. For this reason the appropriate distribution for capturing this event is \(\text{Bernoulli}(1 - p_b)\). An illustration of our model is shown in Figure 1 below.

![Model for purity of a base and sequencing errors](image_url)

**Fig. 1:** Model for purity of a base and sequencing errors.

Of primary interest is to compute the posterior of \(p_b\) given the observed data \(B_i\) conditional on the error parameter \(E_i\), which can be shown to be

\[
p_{\text{obs}} \sim \Pr(p_b|B_i, i = 1, \ldots, r)
\]

\[
\propto \prod_{i=1}^{r} \left[ p_b^{B_i}(1 - p_b)^{1-B_i}(1 - \epsilon) + p_b^{1-B_i}(1 - p_b)^{B_i}\epsilon \right] \times p_b^{\alpha-1}(1 - p_b)^{\beta-1}.
\]

In order to sample \(p_{\text{obs}}\) we must first generate our observed data \(B_i\). To do this we begin by sampling a set of \(r\) sequencing errors \(E_i\) with a fixed error rate \(\epsilon\) (though it should be noted that these errors could be allowed to vary at each position based on quality scores, etc.). With these we then sample our observed data as


\[ B_i \sim \frac{B(\alpha + k, \beta - k + 1)}{B(\alpha, \beta)} = \text{Bernoulli} \left( \frac{\alpha}{\alpha + \beta} \right), \text{if } E_i = 0 \text{ and} \]

\[ B_i \sim \frac{B(\alpha - k + 1, \beta + k)}{B(\alpha, \beta)} = \text{Bernoulli} \left( \frac{\beta}{\alpha + \beta} \right), \text{if } E_i = 1. \]

Here \( B(\alpha, \beta) \) is the beta function which is defined as

\[ B(\alpha, \beta) = \frac{\Gamma(\alpha)\Gamma(\beta)}{\Gamma(\alpha + \beta)}, \]

with \( \Gamma(z) = \int_{0}^{\infty} t^{z-1}e^{-t}dt \) being the gamma function. With the observed samples \( B_i \) we can now use Markov Chain Monte Carlo (MCMC) to sample \( p_{obs} \). For our analysis MCMC was implemented using the \texttt{rjags} package in the \texttt{R} programming language.

**Generating null and alternative distributions**

With a way to sample the observed purities \( p_{obs} \), our objective is to estimate the probability of detecting differences in purities between vials as we vary the true purity \( p_b \) (i.e. the power of the test).

To do this we begin by estimating the null distribution, i.e. when the distribution of \( p_b \) is the same for both vials 1 and 2, specifically \( \alpha_1 = \alpha_2 \) and \( \beta_1 = \beta_2 \), here the subscripts denote the corresponding vials parameters for the Beta distribution (note, this sampling is for a given read depth \( r \)). Recall that the expected value of the beta distribution is \( \frac{\alpha}{\alpha + \beta} \) so that the ratio of these parameters defines the underlying pure probability. In our analysis we consider two scenarios

1. Vial 1 purity \( p_b \) with expectation \( \frac{\alpha_1}{\alpha_1 + \beta_1} = 0.99 \), with vial 2 expectation \( \frac{\alpha_2}{\alpha_2 + \beta_2} \in \{0.98, 0.96, ..., 1\} \).

2. Vial 1 purity \( p_b \) with expectation \( \frac{\alpha_1}{\alpha_1 + \beta_1} = 0.50 \), with vial 2 expectation \( \frac{\alpha_2}{\alpha_2 + \beta_2} \in \{0.48, 0.46, ..., 1\} \).

These are meant to capture the two cases of observed purities we would expect to see in experimental settings.

Given scenarios 1 and 2, let \( p_{obs,j}(1) \) and \( p_{obs,j}(2), j = 1, ..., J \) denote draws from the null distribution for vials 1 and 2 respectively, define \( d^0_j = p_{obs,j}(1) - p_{obs,j}(2) \) to be the difference in the observed purities and \( d^0 = (d^0_1, ..., d^0_J) \) the collection of these differences. In a similar fashion we make draws from the alternative distribution, altering the expectation for vial 2 as defined above; for this case define \( d^1 = (d^1_1, ..., d^1_J) \) as the collection of these differences. With \( d^0 \) and \( d^1 \) we can now estimate the distribution of the differences for the null and alternative hypotheses. To do this we use the binned kernel density estimate function, \texttt{bkde} in \texttt{R}.

**Computing the power of the test**

Pictorially the power of our test, i.e. the probability of detecting a difference when one exists is shown below in Figure 2. Here the starting point of the green region (the power) is determined by the pre-determined level of significance \( \gamma \), i.e. the probability of rejecting the null when no differences exist. Typically this value is taken to be \( \gamma = 0.05 \), but as will be discussed in the
following section, in order to account for multiple comparisons we use an adjustment of this to determine the starting point for our power estimate.

Irrespective the cutoff associated with a particular value of $\gamma$ is established by finding the quantile from the binned kernel density fit to the sampled differences in $d^0$. The area under the alternative distribution for $d^1$ is then computed and corresponds to our estimate of power.

![Fig. 2: Pictorial description of the power to detect significant difference between two distributions.](image)

### Adjusting for multiple comparisons

In order to adjust for multiple comparisons we use the well-known Benjamini-Hochberg (BH) adjustment for controlling False Discovery Rate (FDR). FDR procedures are designed to control the expected proportion of incorrectly rejected null hypotheses, i.e. false discoveries. In contrast, multiple comparison correction using family wise error rate (FWER) based procedures, such as the Bonferroni correction, seek to reduce the probability of even one false discovery, as opposed to expected proportion of false discoveries. Thus FDR procedures have great power at the cost of increased rate false positives.

In its most common form the BH adjustment calls for taking the computed p-values and ordering, smallest to largest as $P_{(1)} \leq P_{(2)} \leq \cdots \leq P_{(G)}$, where $G$ denotes the number of positions in the genome we are computing pairwise comparisons for. The procedure then proceeds by finding the largest $k$ such that $P_{(k)} \leq \frac{k}{G}\gamma$. Intuitively we can think of the proportion $\frac{k}{G}$ as representing the number of pairwise differences we might expect to see correctly rejected. Put another way, if some number of difference are observed between vials at various positions in the genome, this proportion informs how many of those differences we expect to be real versus artifacts of the large number of comparisons or feature of the experimental process. So, for example, taking $\frac{k}{G} = 1.0$ would basically say that we believe all detected differences are real. The impact that this adjustment has on our power estimate is shown in Figure 3.

As the number of actual differences between sequences should be small so to should the ratio $\frac{k}{G}$, however, certain “real” differences do occur so that $k > 0$. So, while many differences may be detected, largely as a result of the massive number of comparisons being made, we believe only a small number of these reflect actual differences. For our purposes we
conservatively set $\frac{k}{G} = 0.05$, i.e. of the differences that are detected, we believe that only 5% may be attributable to real events (and many of these may be related to sequencing artifacts).

Fig. 3: Pictorial depiction of decrease in power to detect differences due to multiple comparisons.