Title: Saliva Samples are a Viable Alternative to Blood Samples as a source of DNA for High Throughput Genotyping.

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Author's response to reviews: see over
Dear Editorial Board,

RE: Submission of Response to Reviewers Comments for Research Article - Saliva Samples are a Viable Alternative to Blood Samples as a source of DNA for High Throughput Genotyping.

The revised manuscript, figure, and additional files have been uploaded. The response to reviewers’ questions is appended to the covering letter. We would like to thank the reviewers for their careful review and their helpful comments. We have addressed each of the issues raised point by point and have made the requisite amendments as per the reviewers’ recommendations where necessary. All text changes within the document are highlighted in red text.

Thank you for considering this article for publication in BMC Medical Genomics.

Yours sincerely,

Dr. J Abraham, Ms. M Maranian, Ms. I Spiteri, Dr. R Russell\textsuperscript{3}, Ms. Susan Ingle, Mr. C Luccarini, Dr. H M Earl, Dr. P Pharoah, Dr. A. Dunning and Professor C Caldas.
All changes or additions to the text of the main manuscript mentioned in response to reviewers’ questions can be found in red in the main manuscript.

Reviewer 1 – Comments

1. **The authors should explain the rationale for doing this study. What are the advantages and disadvantages of saliva sampling in comparison to blood collection?**
   Many of the advantages and disadvantages are already mentioned in the article but we have clarified this further with the following comments in paragraph 1 of the introduction;

   “The potential advantages of saliva sample collection compared with blood sample collection include lower overall cost, lower infection risk, increased patient convenience, acceptability, compliance and uptake. However potential disadvantages include lower mean DNA yield and greater contamination with bacterial DNA. This study investigates the suitability of DNA extracted from saliva compared with DNA extracted from blood for high-throughput genotyping platforms.”

   The cost of transport, inconvenience to the patient and nursing or phlebotomy input to provide a blood sample does not compare favourably with saliva collection despite the cost of the collection kit and associated postal charges.

2. **Is the sampling of unstimulated saliva really easier than blood taking?**

   Several studies have reported that patient preference and compliance rates improve significantly with saliva sampling rather than blood sampling. The co-operation of the patient is paramount to the ease of DNA sample collection. Many manufacturers recommend unstimulated “drool” samples. This is mainly to avoid any of the problems that physical or chemical stimulus used during the collection of saliva may cause, such as absorption or modification of the composition of the saliva. In our study no patients reported experiencing decreased salivary secretion (dry mouth) when asked to provide a sample. We have already addressed the general advantages and disadvantages of saliva and blood sampling in answer to the reviewer’s first question.

3. **What about the potential application of saliva collection devices such as Salivette?**
There are a number of methods available to collect saliva including mouthwash and Salivette. The aim of this article is not to compare saliva collection devices. As mentioned in the previous answers issues have been raised about problems related to absorption and saliva composition.

4. **What was the ratio of bacterial and human DNA isolated from saliva?**

The following sentence has been added to paragraph 2 of the introduction;

“Studies investigating bacterial content in saliva collection samples have estimated that the median bacterial content from Oragene saliva collection kits is 11.8%, whereas bacterial content from mouthwash and buccal swabs was up to 60% and 90% respectively.”

The high concordance and call rates found in this study are further evidence that bacterial DNA content was minimal.

5. **What was the variability of DNA yield?**

The variability of the DNA yield is expressed as the values for range of yield given in Table 1. Whilst the average yield of DNA is considerably lower from saliva, than from blood, the variability of yield is actually greater per ml for blood than for saliva.

6. **Is the yield important for subsequent analyses?**

Both the quality and quantity of DNA is important in future analyses, in that there must be sufficient DNA of adequate quality to fulfil the requirements of the study aims.

As stated, in the results section, the DNA requirements for chip genotyping are 500 nanograms -1 microgram of DNA. The mean DNA yield from saliva in this study was 24 micrograms which is more than adequate to fulfil the requirements of this study.

7. **What about the effects of toothbrushing?**
The instructions on the Oragene package explain this carefully. The instruction includes not eating, drinking, smoking, chewing gum or brushing your teeth for 30 minutes prior to giving the saliva sample. There is reference to the fact that patients were given the collection kit in conjunction with the manufacturer’s instructions. A website link can be added if the editorial team think this is necessary.

8. **Self-collection of saliva is an issue due to the variable volume and quality of the sample. As the volume collected was not assessed the interpretation of the outcomes is difficult and the multiplication by a factor of 2 in comparison to blood questionable. This should be mentioned in the Discussion.**

Although we used the recommended Oragene methodology, we would agree that measuring total volume of sample received would have provided greater confidence that each individual sample was obtained as per the instructions given to the patient i.e. providing 2ml saliva in 2ml preservative. Genotyping on platforms such as the Illumina beadchip requires DNA of a critically specified quantity. Given that the genotype call rate (GCR) of the assessed saliva samples was close to 99% (Table 2 and 3), we are confident that our initial quantifications were accurate. The following sentence has been added to the Results section:

“The study has some limitations. ............... The actual volume of saliva received was not recorded. This value would have provided an estimate of patient compliance with manufacturers’ instructions for using the saliva collection kit and producing the required amount of saliva in the sample. However, the aim of this article was to establish whether DNA of an acceptable quality and quantity could be obtained to allow high-throughput genotyping on different platforms, which we have successfully demonstrated.”

9. **What was the fragmentation of the isolated DNA? Picogreen has some limitations regarding the fragmentation of DNA quantified. The fragmentation should be assessed as it can be important in other applications including sequencing.**

We are not aware of any publications that have observed saliva DNA isolation to be more prone to fragmentation than blood DNA isolation. To evaluate the fragmentation of DNA from both sources, three matched saliva and blood DNA samples were
randomly selected and assessed using the Shimadzu Microchip Electrophoresis System and SYBR®Gold nucleic acid gel stain according to manufacturer’s instructions. Fragmentation was visible for both DNA sources, however, was more marked in DNA isolated from saliva. It is viable that a proportion of this low molecular weight contamination is due to impurities and/or bacteria in the saliva sample. High molecular weight DNA is clearly visible and the majority of low molecular weight contamination observed is < 25bp. Our study has only researched DNA quality for genotyping platforms. Therefore using saliva derived DNA on other platforms such as sequencing would require further investigation.

The following text has been added to the methods section p8 final paragraph:

“Microchip Electrophoresis to assess fragmentation of DNA:

Three matched saliva and blood DNA samples were randomly selected and assessed (see additional files, Supplementary Figure 2) using the Shimadzu Microchip Electrophoresis System and SYBR®Gold nucleic acid gel stain, (2µl DNA in 4µl DNA-500 Marker) according to manufacturer’s instructions.”

The following image has been added to the additional files Supplementary Figure 2:

Assessment of fragmentation of 3 matched blood and saliva derived DNA samples
10. What are the costs of the sampling/isolation? The Oragene kits are not cheap. This should be at least mentioned in the Discussion as a limitation of the use.

There is already reference to the cost of the sampling kits in the discussion which is balanced with the cost of phlebotomy services and transport for the patient to the hospital or community health care provider (p10 final paragraph of the discussion). Both blood and saliva DNA can be commercially extracted or extracted in-house and the difference in cost between the commercial extraction costs is favourable for saliva (Gen-Probe extraction costs for blood £8/sample and for saliva £5.10/sample). The quantification costs are similar for both sample types. If they were quantitated at Gen-Probe it would have cost £1.95/sample. The normalisation would have cost an extra £1.50/sample. If the quantitation and normalisation is completed in-house the consumable and reagent cost is £0.79/sample for quantitation and an extra £0.11 for normalisation.

The following sentence has been added to p10 final paragraph of the discussion;

“In addition commercial extraction of DNA from saliva is cheaper than from blood (Gen-Probe extraction costs for blood £8/sample and for saliva £5.10/sample).”

11. What are the alternatives of sampling and isolation? Could simple whole saliva be used? DNA isolation from saliva is tricky.

Use of simple whole saliva without the use of preservation agents would limit the shelf-life of the sample and the risk of bacterial contamination would be greatly increased. DNA isolation would be essential to exclude non-human DNA which would cause confusion during the interpretation of genotyping results. Other techniques like buccal swabs have already been mentioned in the article.

12. Why did the authors choose the Oragene kits? Are there other alternative options?

At the time the study was initiated Oragene were the most well-established saliva sample collection kit. There alternatives such as salimetrics but again we would
emphasise that this article does not aim to review saliva collection devices. A sentence has been added in the methods section, under saliva extraction as follows:

“Oragene saliva collection device was selected for use because at the time the study was initiated this was the most well-established and commonly used saliva collection system.”

13. The authors should try to compare the results statistically, at least the yield, purity etc.

We have given both the range and the mean values for yield. With regards the purity of the DNA please refer to the information in response to reviewer 2 question 1 and the text below.

In addition the following has been added to the text (p8 Results section, second paragraph):

“The quality of the DNA obtained was assessed by several criteria. Ratios of absorbance at 260nm and 280nm are presented in additional files, Supplementary Table 1. In this study DNA from blood has a mean (A260/A280 ratio) value (1.71), very close to the ideal value of 1.8. The mean (A260/A280 ratio) values from saliva is a little lower (1.56), indicating more protein contamination in the saliva extracted DNA. DNA fragmentation was compared on a bio-analyser and the results are shown in Supplementary Figure 2. Both DNA types have a clear high molecular weight band, but a more intense band of fragmented DNA is visible in DNA extracted from saliva.”

Reviewer 2 – Comments

Major
1. In table 1, the authors need to include a column showing the mean and range for the A260/A280 ratio for blood and saliva-derived DNA. The A260/A280 ratio is a measure of DNA purity and should be reported in order to make meaningful comparisons of the quality of DNA extracted from blood and saliva. Similar
studies comparing the quality and performance of DNA extracted from different sources (blood, saliva, buccal swabs) typically include the A260/A280 ratio.

Ten matched saliva (of varying yields) and blood samples were assessed for A260/A280 (DNA purity), using a CECIL CE2041 (2000 SERIES) spectrophotometer. Purity of saliva DNA was lower than that of DNA derived from blood, with a mean A260/A280 ratio of 1.56 and 1.71 respectively. This is unsurprising given the likelihood and risk of potential contaminants present in the mouth. Quantifying the saliva DNA by Picogreen rather than spectrophotometry ensured the most stringent and accurate yield was obtained.

This table has been provided in the additional files, Supplementary Table 1:

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<td>Blood</td>
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</tr>
<tr>
<td>Mean</td>
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<td>1.71</td>
</tr>
</tbody>
</table>

The following text was added to the methods section p8 paragraph 1:

“Spectrophotometry to assess DNA purity:

Ten matched saliva (of varying yields) and blood samples were assessed for A260/A280 ratio (additional files, Supplementary Table 1). A 1/100 dilution was prepared (DNA/deionised H2O) and analysed, using a CECIL CE2041 (2000 SERIES) spectrophotometer, according to the manufacturers instructions.”

In addition the following was added to the text (p8 Results section, paragraph 2):

“The quality of the DNA obtained was assessed by several criteria. Ratios of absorbance at 260nm and 280nm are presented in additional files, Supplementary Table 1. In this study DNA from blood has a mean (A260/A280 ratio) value (1.71), very close to the ideal value of 1.8. The mean (A260/A280 ratio) values from saliva is a little lower (1.56), indicating more protein contamination in the saliva extracted DNA.
DNA fragmentation was compared on a bio-analyser and the results are shown in Supplementary Figure 2. Both DNA types have a clear high molecular weight band, but a more intense band of fragmented DNA is visible in DNA extracted from saliva.”

2. **DNA Genotek reports that the median bacterial content in DNA samples purified using the Oragene# self-collection kit is 11.8%, therefore the vast majority of DNA is of human origin (http://www.dnagenotek.com/US/pdf/PD-WP-011.pdf). The authors should revise the above statement to incorporate the report on the proportion of bacterial DNA provided by the kit manufacturer.**

   This is a very helpful comment and we have added the following sentence to paragraph 2 of the introduction;

   “Studies investigating bacterial content in saliva collection samples have estimated that the median bacterial content from Oragene saliva collection kits is 11.8%, whereas bacterial content from mouthwash and buccal swabs was up to 60% and 90% respectively.”

3. **In the last sentence of the second paragraph of the background (page 4), the authors discuss a previous study by Bahlo et al. The study assessed the performance of saliva and blood-derived DNA samples on the Illumina genotyping platform, by measuring the concordance of the Genotype Call Rate and Illumina GenCall Score between the paired samples [2]. In reference to this study, the authors state “however no comparison was made of the quality of the data obtained.” This statement is unclear, vague and confusing and needs to be modified to better convey the authors’ point.**

   We have rewritten that sentence to clarify our point. See p4 final paragraph.

   “Another study successfully, used saliva-extracted DNA in a Genome Wide Association Study (GWAS), however there was no clear comparison of the mean DNA yield or range of DNA yield found with each sample type. However the study did show comparable concordance and call rates.”

**Minor**

1. **The authors need to provide table and figure legends.**

   Table and figure titles / legends are provided.

2. **Figure 1 needs to be bigger in order for the axis labels and data labels to be legible.**
Amended as suggested by the reviewer – see Figure 1 document.

3. “DNAgenotek” should be replaced with “DNA Genotek”, which is the way the company name is shown on the company website (http://www.dnagenotek.com/US/company/overview.html).

The changes have been made as suggested by the reviewer.

4. The authors should specify which Gen-Probe genomic DNA extraction kit they used for purification of DNA from blood.

We out-sourced blood DNA extraction to Gen-Probe as stated in both the abstract and methods.

5. There is a typo in the last sentence of the second paragraph on page 4.

The changes have been made as suggested by the reviewer.

6. In the methods section entitled “Saliva DNA extraction” (page 5), the authors state that after collection saliva samples were stored prior to DNA extraction. The authors need to state how long saliva samples were stored and the storage conditions.

The following has been added to the text p6 paragraph 1:

“Patients recruited to this study returned the samples by post or in person, over a period of 2 months from April 2008 to May 2008. Samples were received by a nominated research nurse who stored the samples at room temperature until the samples were brought to the laboratory for extraction. As samples were received at different time points, different samples will have been stored for variable lengths of time.”

The text in the paragraph called “Saliva DNA extraction” already mentions post extraction storage conditions.
7. **The last sentence of the methods section entitled “Taqman Genotyping” (page 6) states that the Taqman assays used in the manuscript were chosen because they were shown to work well in other research projects. The authors need to either provide a reference for this statement or indicate that the data is not shown.**

“Data not shown” has been added

8. **Although this manuscript focuses on human samples, a previous study in dogs compared the performance of paired saliva and blood-derived DNA in dogs using the Illumina Infinium platform and demonstrated that saliva DNA is suitable for high-throughput genotyping studies [1]. The authors should state this in their manuscript, as well as cite the canine study.**

The following sentence has been added to p5 paragraph1:

“One published study in dogs compared the performance of paired saliva and blood-derived DNA in dogs using the Illumina Infinium platform. This study demonstrated that canine saliva DNA was suitable for high-throughput genotyping studies.”

**Discretionary Revisions**

1. **“Additional table” should be changed to “supplemental table” (methods section entitled “Taqman genotyping”) on page 6.**

The formatting instructions from the journal indicate that the extra files should be referred to as “additional”.

So the following change has been made:

“see additional file, Supplementary Table ”