Author's response to reviews

Title: Touchdown General Primer PCR and Optimized Sample DNA Concentration Support the Sensitive Detection of Human Papillomavirus: Investigation of Cervical, VAIN, and Breast Tissues

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RESPONSE TO REVIEWERS’ COMMENTS

REFEREE 1

1. The finding that the GP5+/6+ PCR protocol of de Roda Husman is suboptimal with respect to its sensitivity, is not novel. It should be noted that this initial GP5+/6+ PCR protocol was developed for a water bath PCR apparatus with thick-walled PCR vessels, as was commonly used at that time. Because modern PCR machines heat and cool quite a lot faster, it is not surprising that a general primer method such as this, relying on mismatch acceptance, cannot simply be transferred to a modern PCR machine. This was previously observed by van den Brule et al., who developed a GP5+/6+ protocol for modern PCR machines that gives a sensitivity equal to that of the protocol in the present paper. The paper of van den Brule et al. is cited on page 13 (ref 28) but it would have been appropriate to compare that protocol with the novel touchdown protocol described here.

Response:
The type of thermal cycler used by de Roda Husman et al. in their 1995 paper detailing the GP5+/GP6+ assay is not indicated in the publication. The modified ramping time (MRT) version of the assay developed by van den Brule et al. (2002) is mentioned in a publication entitled “GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes.” Neither in this title nor in the abstract is there anything to indicate that the paper details improved PCR cycling conditions for use with the GP5+/GP6+ primers; we did not become aware of this protocol until after the completion of the study. Further, the paper does not actually give any details of what improvement to HPV detection is conferred by this protocol. The only thing that is said is “The modified ramping times between the temperatures used for denaturation, annealing, and elongation appeared essential for optimal PCR.” There does not appear to have been a specific publication detailing how the development of the protocol, what the improved sensitivity is, its performance relative to the standard conditions, gel/Southern blot data, effect of background DNA, etc.

We have now adapted the MRT protocol (which features slow ramping between PCR steps – not fast times) to include hot-start, dUTP, and 50 cycles of PCR and have tested the assay on selected samples. Firstly, the method was tested on SiHa cell DNA diluted in C-33A DNA. The data shows the MRT method detects HPV-16 with similar efficiency to a touchdown protocol. Secondly, the MRT protocol was tested on samples that were HPV negative by the GP5+/GP6+ assay, but positive with the TDP3 assay, and on samples negative by the GP5+/GP6+ and TDP3 methods. With reference to these additional tests the following text is now included in the revised manuscript:

Methods:
Following the completion of the study the authors became aware of a GP5+/GP6+ protocol incorporating modified ramping times (MRT) [13]. The MRT GP5+/GP6+ conditions were tested on a subset of the samples. For the purposes of this study, the protocol was modified to include hot-start, dUTP, and 50 PCR cycles. Reaction mix was as above. The MRT GP5+/GP6+ cycling conditions are as follows:

HK™-UNG incubation step: 37°C 30 mins
Denaturation/HotStarTaq activation: 95°C 15 mins
Cycles (n=50): 20s at 94°C
In 24 s to 90°C
In 66s to 48°C
In 30s to 38°C
30s at 38°C
In 18s to 42°C
Results:
The MRT GP5+/GP6+ protocol was also tested on SiHa cell DNA diluted in a background of 100ng C-33A DNA and supported detection of HPV-16 from 0.1ng SiHa cell DNA. Figure 2 details a Southern blot of PCR products from the TDP3 and MRT protocols hybridized with a biotin-labeled HPV-16 oligonucleotide probe.

And,

Fifty-four of the 65 samples were also tested with the MRT protocol. Of these 54 samples, 10 were HPV negative and 44 were HPV positive by the TDP3 protocol. Of the 10 negative samples, 1 tested HPV-53 positive by the MRT method. Of the 44 'TDP3 protocol' positive samples, 16 (36%) were negative with the MRT method. The HPV types that went undetected in this sample were types: 16, 18, 31, 35, and 81. An additional set of 25 abnormal cytological samples that tested negative by the TDP3 assay were identified. None of the samples tested HPV positive after PCR with the standard GP5+/GP6+ protocol (50 cycles PCR). Two of the 25 samples tested positive by the MRT protocol (for HPV-39, and for HPV-51). Thus, of 44 samples negative with the standard GP5+/GP6+ assay, 44 (100%) tested positive with the TDP3 protocol, and 28 (64%) tested positive with the MRT conditions; and, of 35 samples negative by the standard GP5+/GP6+ assay and the TDP3 assay, 3 (8.6%) tested positive with the MRT protocol.

Discussion:
The MRT GP5+/GP6+ protocol was also found to detect low-copy HPV in a background of human sequences. This assay incorporates periods of slow temperature changes from the denaturation step to the annealing step and from the annealing step to the extension step. It can be envisaged that this approach may allow for better specific annealing/extension of primers with HPV target during the slow descent to the final annealing temperature of 38°C than occurs with the standard conditions.

And,

The MRT GP5+/GP6+ conditions were tested on 54 of the 65 GP5+/GP6+ negative samples. Twenty-eight (64%) of 44 samples positive by the TDP3 were also positive by the MRT protocol, and 1 of 10 samples negative by the TDP3 protocol was positive by the MRT. Of an additional 25 abnormal cytological samples negative for HPV by the TDP3 assay, none tested positive with the standard GP5+/GP6+ method, but two samples were positive by the MRT protocol. These data again show that HPV detection can be highly dependent on assay conditions and also demonstrate that two or more methods may be required for inclusive HPV screening.

2. The confirmation of PCR product specificity should be done by Southern blotting after agarose gel electrophoresis of PCR products, instead of the dot blotting used here, because the latter does not give information about the product size.

Response:
A Southern blot was performed on SiHa cell DNA contained in C-33A DNA of PCR product from the TDP3 assay and from the MRT assay. See Figure 2.
3. The percentage of breast cancer samples found positive for HPV is very high. Although carry-over contamination control is done by UNG, sample cross-contamination cannot be excluded. Therefore, paraffin sectioning blanks and isolation blanks should be included.

Response:
de Villiers, Sandstrom, zur Hausen, and Buck have recently published data demonstrating HPV (anogenital or skin types) in 89% of breast tumors. In the light of this finding, the estimate of 58% is not so high. This publication is now cited.
The following has been added to the Control Measures section of the Methods:
  FFPE tissue-block sectioning: Measures to prevent potential cross-contamination of tissue during sample sectioning, included wiping the microtome blade with histoclear (xylene substitute) and ‘DNA-Erase’ [ICN] (a DNA contamination removal reagent) between blocks. Additionally, the first 10-20 sections cut from a specimen were discarded prior to collecting sections for DNA extraction from the specimen.

4. Discretionary changes:
   Figure 1 has been deleted.

REFEREE 2

1. I don't like the title, given that the role of HPV in breast cancer is uncertain at best. The authors make this point clear in the discussion but this is a long paper, etc. I think that the title could be much more conservative and still convey the main objective of the paper.

Response:
The title has been changed to the following that indicates the main finding and which is also descriptive of what was done:

  Touchdown General Primer PCR and Optimized Sample DNA Concentration Support the Sensitive Detection of Human Papillomavirus: Investigation of Cervical, VAIN, and Breast Tissues

2. The authors need to better emphasize that increased detection is not necessarily useful in clinical applications. Since current tests already detect the bulk of precancer and cancer, the increased analytic sensitivity cannot help clinical sensitivity but can reduce clinical specificity.

Response:
The following has been added to the Abstract:
The clinical utility of a touchdown approach for HPV detection requires further investigation as increased assay analytical sensitivity may not necessarily equate with improved clinical sensitivity or specificity.

The Discussion now includes a section Clinical Utility:

The impetus for this study was to determine whether abnormal cytological samples that tested HPV negative by a commonly used PCR assay were in fact HPV positive. Using a touchdown protocol, HPV has been demonstrated in a high percentage of samples previously recorded as HPV negative consistent with HPV representing a necessary cause of most (if not all) abnormal cervical cytological conditions. However, an assay with high analytical sensitivity for HPV might not be appropriate as a clinical test. The HPV status of cytological samples has been proposed as a marker to identify patients with underlying high-grade cervical intraepithelial neoplasia (CIN) [30]
and current HPV tests detect the bulk of high-grade CIN and invasive cervical carcinoma. Increased sensitivity for HPV might lead to reduced test specificity for high-grade CIN.

3. One limitation that should be discussed is that they did not address assay reliability.

**Response:**
Assay reliability is now referred to in the Discussion:
The MRT GP5+/GP6+ conditions were tested on 54 of the 65 GP5+/GP6+ negative samples. Twenty-eight (64%) of 44 samples positive by the TDP3 were also positive by the MRT protocol, and 1 of 10 samples negative by the TDP3 protocol was positive by the MRT. Of an additional 25 abnormal cytological samples negative for HPV by the TDP3 assay, none tested positive with the standard GP5+/GP6+ method, but two samples were positive by the MRT protocol. These data again show that HPV detection can be highly dependent on assay conditions and also demonstrate that two or more methods may be required for inclusive HPV screening. Nevertheless, the above comparison of methods together with the demonstration of HPV in 94% of 799 abnormal cytological samples indicates a touchdown approach supports reliable and sensitive HPV detection.

4. I guess that this is the advantage of an on-line article but there seemed to be a great number of figures, etc.

**Response:**
One figure has been deleted – but another added.

5. I do not agree with the approach of testing only HPV- abnormal cytology specimens to determine if an assay is better than another---a number of papers have urged against this approach because of potential bias. It is likely that if we were to test "touchdown" negative specimens by the standard GP5+/6+, some would have tested positive. Or, GP5+/6+ positives might have tested negative by "touchdown". A proper approach would be to retest all specimens or a stratified sample of specimens in a masked fashion. Then present to the readers a two by two table with Kappa stats and McNemar's test. Secondarily, details about which types were missed, etc.

**Response:**
It was not practicable to retest all specimens. However, a set of 25 samples that were negative following primary screening by the touchdown approach were identified. None of these tested positive with the standard GP5+/GP6+ assay, two were positive by the MRT method. The inclusion of these data in the manuscript is detailed in response to Reviewer 1 comments.

6. An additional concern is that the FFPE tissues could have been contaminated when they were cut, leading to elevated HPV positivity by the more sensitive touchdown assay. What PCR precautions were taken? Did the authors run any tissues that are known to be HPV-? For example, I do not think that anyone would attribute brain cancer to HPV infection.

**Response:**
The PCR precautions are detailed in response to Referee 1. Run blanks and C-33A cell line DNA have been used as negative controls, but FFPE tissues were not used as negative controls.