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BRIEF REPORT

Clinical validation of a novel real-time human papillomavirus assay for simultaneous detection of 14 high-risk HPV type and genotyping HPV type 16 and 18 in China

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Abstract In the present study, we describe the laboratory workflow and the clinical validation of a novel multiplex real-time PCR-based HPV assay in China. The cross-sectional validation analysis showed that this assay worked well for detection of 14 HR-HPV types and identification of HPV 16 and 18 in a single sensitive assay that is suitable for both clinical usage and high-throughput cervical screening purposes. We predict that this accurate, high-throughput and low-cost HPV assay can greatly reduce the heavy economic burden of HPV detection in China.

Worldwide, cervical cancer is the second most common cancer in women. Approximately 200,000 women die of this disease each year [1, 2]. Persistent infection by high-risk oncogenic human papillomavirus (HPV) types is now recognized as the necessary cause of cervical cancer [3]. HPV DNA testing, because of its greater sensitivity for cervical precancer and cancer than cytology with one screening, can provide 60–70 % greater reassurance for women who are found negative by screening than can be offered by cytology [4–7]. Therefore, the use of an HPV test has been suggested for primary screening, the triage of women with equivocal cytological results, and the follow-up of patients after treatment for high-grade cervical intraepithelial neoplasia (CIN 2+) [8, 9].

Currently, no "gold standard" HPV detection methods have been defined, but guidelines for their validation have been developed, and quality assurance programs are being performed [10–12]. Suggested quality standards for HPV tests used in clinical testing included the criterion that the test should target at least 13 high-risk HPV (hrHPV) types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and, ideally, HPV type 66 as well. Among the hrHPV types, infections with types 16 and 18 confer the highest risk for persistence and the subsequent development of cervical cancer [13, 14]. Therefore, the American National Comprehensive Cancer Network clinical guidelines regarding screening for cervical cancer and the American Society for Colposcopy and Cervical Pathology (ASCCP) have highlighted the need for specific identification of HPV types 16 and 18 in those with hrHPV-positive populations [9].

China has nearly a quarter of the world's population. Although the incidence of cervical cancer in China is low in comparison to that of western countries, China faces a heavy burden of cervical cancer. Annually, there are about 500,000 new cervical carcinoma cases worldwide and about 131,500 (29 %) cases in China [1, 15]. Therefore, it is of great significance to develop an accurate, high-throughput and cost-effective HPV assay for management of patients with HPV infection and for cervical cancer screening in China.

A wide variety of assays are now available for HPV detection, most of which do not distinguish between the 14 targeted HR types. Three genotyping assays have been approved by the U.S. Food and Drug Administration, but the recently approved cobas test (Roche Molecular Systems Inc., Alameda, CA, USA) is the only one that performs simultaneous HR HPV detection and identification

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of types 16 and 18 using the multiplex real-time technology [8]. The Hybribio 21 HPV GenoArray assay (Chaozhou Hybribio Limited Corp, Chaozhou, China), which identifies 13 hrHPVs (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68), five low-risk HPVs (lrHPVs) (6, 11, 42, 43, 44) and HPV types 53, 66 and CP8304, which are common in the Chinese population, is one of the most commonly used gene chip assays for testing the cervical scraping specimens for clinical HPV genotyping in China. However, this assay is generally considered unsuitable for cervical cancer screening because it is expensive and difficult to automate due to the complicated post-PCR analysis required.

As a consequence, a novel HPV assay has been designed and released by HybriMax (Hybribio Limited, Chaozhou, China), following the updated international guidelines for HPV management of HPV infection and cervical cancer screening [9]. Similar to the cobas 4800 HPV test, the novel assay described here employs multiple real-time technology for simultaneous detection of the 14 hrHPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and identification of HPV types 16 and 18 in a high-throughput setting. To the best of our knowledge, this HPV assay is the first self-developed commercial kit available for simultaneous detection of 14 high-risk HPV genotypes with HPV16/18 genotyping in China. The present paper describes the laboratory workflow and clinical validation of the assay.

The multiplex real-time 12 + 2 HPV test kit was designed and developed by Chaozhou Hybribio Limited Corporation (Hybribio Limited, Chaozhou, China). An overview of the sequences of the primers and fluorescent probes is shown in Table 1. Target HPV DNA was amplified using primers that target the E region of the genome. Fluorescently labeled cleavage probes were used for detection of amplification products (size: 70-200 bp). The designed primers and probes were made by Invitrogen Limited Corporation (Guangzhou, China). The primers and probes could be classified into four groups. Probes and primers in group 1 were used for general detection of the 12 non-HPV16/HPV18 HR-HPV (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) genotypes. Probes and primers in group 2 for HPV 16, group 3 for HPV 18, and group 4 for β-globin served as internal controls to determine the quality of the sample DNA and the presence of potential inhibitory substances. All of the probes were labeled with fluorophores. In total, four different fluorophores were used: HEX for HPV 16, ROX for HPV 18, FAM for 12 non-HPV16/HPV18 hrHPV genotypes, and Cy5 for β-globin.

The blank control was distilled water without a DNA sample. The negative control was female human DNA that was confirmed to be HPV negative. The positive control was a series of constructed plasmids for each of the 14 HPV types mixed with female human DNA. The optimization of each multiplex PCR was based on plasmids for each HPV type and female human DNA. Numerous experiments were performed to evaluate the primers, probes, fluorochromes, cycling parameters and the effect of background DNA. No cross-amplification of any of the type-specific primers with the other HPV types was observed.

We then compared the detection of hrHPV using the new real-time HPV genotyping kit to that using the Hybribio 21 HPV GenoArray test in a routine clinical diagnostic setting. Samples that gave discrepant test results with these two HPV assays were analyzed by direct sequencing. The procedure for the clinically used GenoArray assay (Chaozhou Hybribio Limited Corp, Chaozhou, China) has been detailed in previous publications [16, 17]. For the new real-time assay, DNA was isolated from another 200 µl of each swab sample by the alkaline lysis method (Hybribio Biotechnology Limited Corp.) and eluted in 100 µl of water. Five µl of the DNA solution was used for PCR amplification using the real-time 12 + 2 HPV test kit. Each reaction mixture (25 µl) consisted of 3 µl of Taq DNA polymerase, 17 µl PCR MIX and 5 µl of temple DNA. The PCR and post-PCR analysis were performed in an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The reaction conditions for PCR were 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s and 60 °C for 60 s. The fluorescence signal was acquired at the annealing step of each cycle during amplification using four detection channels: FAM (518 nm), HEX (553 nm), ROX (607 nm), and Cy5 (667 nm). Each PCR run contained both the sample and a control. A sample was considered positive if the amplification curve occurred in at least one of the three predefined type-specific detection channels (FAM, HEX, or ROX) and the control (Cy5: β-globin) were also positive. The genotype was differentiated according to the specific channel at which amplification could be detected.

A total of 1050 cervical samples with validated HPV genotyping results, including 300 cases from the First Hospital Affiliated to Jinan University, 350 cases from Sun yat-sen Memorial Hospital of Sun yat-sen University, and 400 cases from the Guangdong Women and Children's Hospital, were tested using the Hybribio 12 + 2 hrHPV test according to recommendations of the manufacturer (HybriMax) in a blinded fashion. Each sample was analyzed in duplicate. All of the clinical samples were liquid-based cytology samples that were collected from a large population of women who were referred to the three above-mentioned hospitals for opportunistic screening, triage of abnormal cervical cytology, or follow-up after treatment of cervical intraepithelial neoplasia from December 2010 to March 2012. HPV results with the 21 HPV GenoArray test

Table 1 The sequences of the primers and fluorescent probes used in the multiplex PCRs

Multiplex PCRs	Primer or probe	Sequence (5'-3')
1 12HR-HPV (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68)	HPV31 Probe	CTGTCTGTCTGTCA
	HPV31 Primer-F	GAGCACACAAGTAGATATTC
	HPV31 Primer-R	GTCCTCTGAAATGTTGTC
	HPV33 Probe	TGACATACAGACAGACA
	HPV33 Primer-F	CGTCTATATCTAGCAACCA
	HPV33 Primer-R	GCTGTTCTATTGTCCAAG
	HPV35 Probe	TCTACATCTGACTGC
	HPV35 Primer-F	TGGACAGTGTTGACAGAG
	HPV35 Primer-R	CCCAATCTATATCTTGAACACTTTA
	HPV39 Probe	CACTGCTGTCTGTAT
	HPV39 Primer-F	GACCTGGCAGACTTTATTG
	HPV39 Primer-R	CCTGGTATTCCTGCCTAC
	HPV45 Probe	CATAGACAGACTGC
	HPV45 Primer-F	CGGTGGGATACATGACTA
	HPV45 Primer-R	CCAACAACCAAGCAAAAG
	HPV51 Probe	ACACAGCCATAGTC
	HPV51 Primer-F	GCCCATTAGGAGACATTA
	HPV51 Primer-R	CGGATAACTGTCCAGTAA
	HPV52 Probe	CACTGCTGCTGTCA
	HPV52 Primer-F	GCATCTGGTCATGTATTG
	HPV52 Primer-R	CCTGGAATAGGTGTACTAC
	HPV56 Probe	TTCAACAATCCACAGG
	HPV56 Primer-F	AAGGCAGCTTATTCTGTG
	HPV56 Primer-R	CTCAGCACGTGTTAGTTC
	HPV58 Probe	CTATCGTCTGCTGT
	HPV58 Primer-F	AGCGGTAATAGAACGAAGA
	HPV58 Primer-R	CTGTGTAGTACTTTGTACTGAATC
	HPV59 Probe	ACAGCGTATCAGCAGC
	HPV59 Primer-F	GCAAGAGTAAGAGAATTAAGA
	HPV59 Primer-R	GTTGGACATAGAGGTTTTAG
	HPV66 Probe	TGTCTACTCGTATGTCT
	HPV66 Primer-F	CGATGTCAATGTCCGTTA
	HPV66 Primer-R	GCATGGTTATACTGTAGATTC
	HPV68 Probe	TCACTGTCATCTGT
	HPV68 Primer-F	CATGGAATAGATGATAGTGTA
	HPV68 Primer-R	GCAGCATTACTATTACAATC
2 HPV16	HPV16 Probe	CACTATCGTCTACTACTATGTCA
	HPV16 Primer-F	ACAGGTATATCAAATATTAGTGAA
	HPV16 Primer-R	CTTGCATTACTATTAGTGTCTG
3 HPV18	HPV18 Probe	TCTATGTCACGAGC
	HPV18 Primer-F	AGCCCCAAAATGAAATTC
	HPV18 Primer-R	CACACTTACAACACATACA
4 β-globin	β-globin Probe	TGCTTCTGACACAACT
	β-globin Primer-F	GAGCCATCTATTGCTTACA
	β-globin Primer-R	CTCACCACCAACTTCATC

and cytology data of these scrapings were retrieved from the trial database. Because the data were analyzed anonymously and specimens for this study were used after the completion of clinical diagnostic work, the hospital ethics committees approved a waiver of written consent.

Of the 1050 eligible women, 39.0 % (410/1050) tested positive for high-risk HPV infection by the GenoArray test. Forty-six women were diagnosed with CIN1, 23 with CIN2 and 21 with CIN 3 or worse. Sixty-seven women had abnormal cytology of ASC-US or worse (24 cases with ASC-US, 1 case with ILIS, 1 case with HSIL, 10 cases with squamous cell carcinoma, and 31 cases with atypical glandular cells).

A valid real-time PCR test result was obtained for all of the 1050 liquid-based cytology samples. Agreement between the 12 + 2 hrHPV real-time PCR test and HPV GenoArray test was strong for the 14 pooled high-risk types as well as for each HPV type (Tables 2 and 3). For detection of all 14 hrHPV types without distinguishing between them, the overall concordance between the two assay methods reached 96.48 % (1013/1050, 95 % CI: 95.36 %-97.59 %), with a kappa value of 0.927 (95 % CI: 91.1 %-94.2 %), while the 12 + 2 real-time PCR test provided a significantly larger number of hrHPV-positive results than the HPV GenoArray test (41.24 % vs. 39.8 %, $p < 0.001$). For the specific genotyping assay, a total of 47 specimens showed discordant genotyping results for the two methods. The overall genotyping agreement for the two HPV tests was 95.52 % (1003/1050), with a kappa value of 0.906 (95 % CI: 0.847-0.965). Furthermore, there was no significant difference in the positive detection rates between the two methods (40.28 % vs. 39.8 %, $p > 0.05$).

Forty-seven specimens (47/1050) for which the two tests yielded discrepant genotyping results were further analyzed by direct sequencing. Of the seven GenoArray test-positive/real-time PCR test-negative specimens, three were confirmed HPV positive by direct sequencing (one case of HPV33, one case of HPV58 and one case of HPV68), and the remaining four were determined to be HPV negative. Of the 30 GenoArray test-negative/real-time PCR test-positive specimens, 15 (7 cases of HPV51, 3 cases of HPV16, 2 cases of HPV66, 1 case of HPV18, 1 case of HPV39, and 1 case of HPV39/45/51) were positive by sequencing, and the other 15 specimens were negative. In the real-time PCR test, 14 of the 15 specimens that were shown to be negative by sequencing were found to be infected with other hrHPV types, and one was infected with HPV16. The accuracy of the real-time PCR assay vs. the GenoArray test for this misdiagnosis samples was 51.4 % vs. 48.6 % for general hrHPV detection and 55.3 % vs. 44.7 % for genotype determination.

With regard to the study subjects with abnormal histology results and cytology interpretation, the 12 + 2 hrHPV real-time PCR test and GenoArray test showed no significant difference in the rates of detection of the 14 HR-HPV genotypes in samples with CIN1 and CIN2 or worse or in the different cytology interpretation groups ($p > 0.05$). Recently, Hesselink et al. [18] conducted a clinical validation analysis of a very similar novel HPV-Risk assay (Self-Screen BV, Amsterdam, The Netherlands). The agreement of the HPV-Risk assay was also high when compared in a non-inferiority study with GP5/6 PCR, for both self-collected (cervical) vaginal specimens and clinician-obtained cervical scraping specimens. The data support further evaluation of the clinical performance of our novel 12 + 2 high-risk HPV real-time assay in cervical cancer screening protocols. In conclusion, the

Table 2 Comparison of the Hybribio 12 + 2 HPV real-time PCR assay and the HPV GenoArray test using 1,050 liquid-based cervical smears[a]

Hybribio 12 + 2 HR-HPV test results	No. of specimens analyzed by Hybribio 21 gene chip test								Total
	Negative	Positive							
		16	18	16/18	Other hrHPV	16 and other hrHPV	18 and other hrHPV	16/18 and other hrHPV	
HPV negative	610				7				617
HPV16	4	73							77
HPV18	1		13						14
HPV16/18			1	2					3
Other hrHPV	25				271	1			297
16 and Other hrHPV		5			1	31			37
18 and Other hrHPV			2				2		4
16/18 and other hrHPV								1	1
Total	640	78	16	2	279	32	2	1	1050

[a] Overall HPV test agreement (i.e., hrHPV negative/positive) of 96.48 % (1013/1050 specimens; 95 % CI), Kappa value, 0.927 ($p = 0.000$) Genotype agreement (i.e., HPV16, -18, others) of 95.52 % (1003/1050 specimens, 95 % CI, 94.2 %-96.7 %), Kappa value of 0.906 ($p = 0.0000$)

Table 3 Agreement between the Hybribio 12 + 2 hrHPV test and the 21 HPV GenoArray test for pooled detection and genotyping of 14 hrHPV type

12 + 2 hrHPV real-time PCR test results	No. of specimens analyzed by 21 HPV gene chip test that are HPV:		Agreement (%)	Kappa value (95 % CI)	McNemar *p*-value
	Negative 640 (60.9 %)	Positive 410 (39.1 %)			
Pool detection (hrHPV negative/positive)					
Negative (617, 58.76 %)	610 (58.1 %)	7 (0.67 %)	96.5 % (95.4-97.6)	0.927 (0.91-0.94)	0.000
Positive (433, 41.24 %)	30 (2.9 %)	403 (38.4 %)			
Genotyping[a] (-16, -18, others)					
Negative (627, 59.42 %)	610 (58.1 %)	17 (1.6 %)[b]	95.5 % (94.2-96.7)	0.906 (0.85-0.96)	0.079
Positive (423, 40.28 %)	30 (2.9 %)	393 (37.4 %)			

[a] Samples that tested positive by both the Hybribio 12 + 2 hrHPV test and the 21 HPV GenoArray test but with discrepant genotype results are classified into the Hybribio 12 + 2 hrHPV test (-)/GenoArray test (+) group

described multiplex real-time PCR-based HPV assay allows simultaneous detection of the 14 hrHPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and identification of HPV types 16 and 18 in a high-throughout setting. The cross-sectional validation analysis clearly indicated that the clinical accuracy of the novel 12 + 2 hrHPV real-time PCR assay was not inferior to that of the clinically validated 21 GenoArray test. Improvement of the novel 12 + 2 hrHPV real-time PCR assay is still needed, i.e., a few cases of HPV infection could not be using only one of the test kits or were falsely positive, requiring additional analysis (DNA sequencing). Nevertheless, the new test can serve as a rapid primary screening method for management of women with HPV infection and for cervical cancer screening. Moreover, the cost of the novel multiplex real-time PCR-based HPV assay was much lower than that of the currently clinical widely used HPV GenoArray assay. We predict that this accurate, high-throughput and low-cost HPV assay can greatly reduce the heavy economic burden of HPV detection in China.

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Compliance with ethical standards

Conflict of interest LY Yang provides occasional consultation to Chaozhou Hybribio Limited Corp. LX Xie and LJ Li are on the scientific advisory board of Hybribio. All other authors declare that they have no conflict of interest. The HPV test reagents were kindly provided by Hybribio; the funding sources did not have any influence on the design of the study or on analysis of the results.

References

1. Arbyn M, Castellsagué X, de Sanjosé S, Bruni L, Saraiya M, Bray F, Ferlay J (2011) Worldwide burden of cervical cancer in 2008. Ann Oncol 22:2675–2686
2. Forouzanfar MH, Foreman KJ, Delossantos AM, Lozano R, Lopez AD, Murray CJ, Naghavi M (2011) Breast and cervical cancer in 187 countries between 1980 and 2010: a systematic analysis. Lancet 378:1461–1484
3. Trottier H, Burchell AN (2009) Epidemiology of mucosal human papillomavirus infection and associated diseases. Public Health Genomics 12:291–307
4. Anttila A, Kotaniemi-Talonen L, Leinonen M, Hakama M, Laurila P, Tarkkanen J, Malila N, Nieminen P (2010) Rate of cervical cancer, severe intraepithelial neoplasia, and adenocarcinoma in situ in primary HPV DNA screening with cytology triage: randomised study within organised screening programme. BMJ 340:c1804
5. Bulkmans NW, Berkhof J, Rozendaal L, van Kemenade FJ, Boeke AJ, Bulk S, Voorhorst FJ, Verheijen RH, van Groningen K, Boon ME, Ruitinga W, van Ballegooijen M, Snijders PJ, Meijer CJ (2007) Human papillomavirus DNA testing for the detection of cervical intraepithelial neoplasia grade 3 and cancer: 5-year follow-up of a randomised controlled implementation trial. Lancet 370:1764–1772
6. Ronco G, Giorgi-Rossi P, Carozzi F, Confortini M, Dalla Palma P, Del Mistro A, Ghiringhello B, Girlando S, Gillio-Tos A, De Marco L, Naldoni C, Pierotti P, Rizzolo R, Schincaglia P, Zorzi M, Zappa M, Segnan N, Cuzick J, New Technologies for Cervical Cancer screening (NTCC) Working Group (2010) Efficacy of human papillomavirus testing for the detection of invasive cervical cancers and cervical intraepithelial neoplasia: a randomised controlled trial. Lancet Oncol 11:249–257
7. Sankaranarayanan R, Nene BM, Shastri SS, Jayant K, Muwonge R, Budukh AM, Hingmire S, Malvi SG, Thorat R, Kothari A, Chinoy R, Kelkar R, Kane S, Desai S, Keskar VR, Rajeshwarkar R, Panse N, Dinshaw KA (2009) HPV screening for cervical cancer in rural India. N Engl J Med 360:1385–1394
8. Laudadio J (2013) Human papillomavirus detection: testing methodologies and their clinical utility in cervical cancer screening. Adv Anat Pathol 20:158–167
9. Saslow D, Solomon D, Lawson HW et al (2012) American Cancer Society, American Society for Colposcopy and Cervical Pathology, and American Society for Clinical Pathology screening guidelines for the prevention and early detection of cervical cancer. Am J Clin Pathol 137:516–542
10. Meijer CJ, Berkhof J, Castle PE, Hesselink AT, Franco EL, Ronco G, Arbyn M, Bosch FX, Cuzick J, Dillner J, Heideman DA, Snijders PJ (2009) Guidelines for human papillomavirus

DNA test requirements for primary cervical cancer screening in women 30 years and older. Int J Cancer 124:516–520
11. Stoler MH, Castle PE, Solomon D, Schiffman M, American Society for Colposcopy and Cervical Pathology (2007) The expanded use of HPV testing in gynecologic practice per ASCCP-guided management requires the use of well validated assays. Am J Clin Pathol 127:335–337
12. Eklund C, Zhou T, Dillner J, WHO Human Papillomavirus Laboratory Network (2010) Global proficiency study of human papillomavirus genotyping. J Clin Microbiol 48:4147–4155
13. Khan MJ, Castle PE, Lorincz AT, Wacholder S, Sherman M, Scott DR, Rush BB, Glass AG, Schiffman M (2005) The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. J Natl Cancer Inst 97:1072–1079
14. Kjær SK, Frederiksen K, Munk C, Iftner T (2010) Long-term absolute risk of cervical intraepithelial neoplasia grade 3 or worse following human papillomavirus infection: role of persistence. J Natl Cancer Inst 102:1478–1488
15. Kim K, Zang R, Choi SC, Ryu SY, Kim JW (2009) Current status of gynecological cancer in China. J Gynecol Oncol 20:72–76
16. Lin M, Yang LY, Li LJ, Wu JR, Peng YP, Luo ZY (2008) Genital human papillomavirus screening by gene chip in Chinese women of Guangdong province. Aust N Z J Obstet Gynaecol 48:189–194
17. Chen Q, Xie LX, Qing ZR, Li LJ, Luo ZY, Lin M, Zhang SM, Chen WZ, Lin BZ, Lin QL, Li H, Chen WP, Zheng PY, Mao LZ, Chen CY, Yang C, Zhan YZ, Liu XZ, Zheng JK, Yang LY (2012) Epidemiologic characterization of human papillomavirus infection in rural Chaozhou, eastern Guangdong Province of China. PLoS One 7:e32149
18. Hesselink AT, Berkhof J, van der Salm ML, van Splunter AP, Geelen TH, van Kemenade FJ, Bleeker MG, Heideman DA (2014) Clinical validation of the HPV-risk assay, a novel real-time PCR assay for detection of high-risk human papillomavirus DNA by targeting the E7 region. J Clin Microbiol 52:890–896