Title: Evaluation of the suitability of RNAscope as a technique to measure gene expression in clinical diagnostics: a systematic review.

Running Heading: Evaluation of RNAscope technique for gene expression in clinical diagnostics: A systematic review

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Declarations:

Funding: No funding was received for the research or the manuscript.

Conflicts of interest/Competing interests: The authors declare no conflicts of interest.

Availability of data and material (data transparency): All the data are available from the corresponding author upon request.

Authors' contributions

- Sameeha Atout (conceived and designed the analysis, collected the data, contributed data and analysis tools, performed the analysis, wrote the paper)
- Shaymaa Shuraab (collected the data, contributed data and analysis tools, performed the analysis, reviewed and edited the manuscript)
- Carolyn Loveridge (the senior author who supervised the whole research project, participated in developing the idea, reviewed and edited the manuscript)

Acknowledgements:

The team of authors acknowledge Dr. Shameq Sayeed and Dr Tomoko Iwata for their helpful discussions and for commenting on the manuscript.

Abstract

Objective: To evaluate the application of RNAscope in the clinical diagnostic field compared to the current 'gold standard' methods employed for testing gene expression levels, including immunohistochemistry (IHC), quantitative real time PCR (qPCR), and quantitative reverse transcriptase PCR (qRT-PCR), and to detect genes, including DNA in situ hybridisation (DNA ISH).

Methods: This systematic review searched CINAHL, Medline, Embase, and Web of Science databases for studies that were conducted after 2012 and that compared RNAscope with one or more of the 'gold standard' techniques in human samples. QUADAS-2 test was used for the evaluation of the articles' risk of bias. The results were reviewed narratively and analysed qualitatively.

Results: A total of 27 articles (all retrospective studies) were obtained and reviewed. The 27 articles showed a range of low to middle risk of bias scores, as assessed by QUADAS-2 test. Twenty-six articles studied RNAscope within cancer samples. RNAscope was compared to different techniques throughout the included studies (IHC, qPCR, qRT-PCR and DNA ISH). The results confirmed that RNAscope is a highly sensitive and specific method that has high concordance rate (CR) with qPCR, qRT-PCR, and DNA ISH (81.8% - 100%). However, the CR with IHC was lower than expected (58.7% - 95.3%), which is mostly due to the different products that each technique measures (RNA vs. protein).

Discussion: This is the first systematic review to be conducted on the use of RNAscope in the clinical diagnostic field. RNAscope was found to be a reliable and robust method that could complement gold standard techniques currently used in clinical diagnostics to measure gene expression levels or for gene detection. However, there was not enough data to suggest that RNAscope could stand alone in the clinical diagnostic setting, indicating further prospective studies to validate diagnostic accuracy values, in keeping with relevant regulations, followed by cost evaluation are required.

Keywords: mRNA, RNAscope, immunohistochemistry (IHC), RT-PCR, qRT-PCR, DNA ISH, sensitivity, specificity, Concordance rate (CR)

Key Points:

1. RNAscope is a novel technology that can be used to measure gene expression (RNA).

2. RNAscope could be used as a complementary technique alongside existing procedures to enhance the diagnosis of disease that occurs as a result of abnormal gene expression, for example to confirm any unclear results from gold standard methods.

3. For RNAscope to be used as a tool to diagnose disease, further research is required to fully validate the technique so that it complies with regulatory standards and to assess cost implications for the health service.

1. Introduction

1.1 The developmental history of RNAscope

Gene expression involves transcription of DNA into messenger RNA (mRNA) followed by translation of mRNA to protein. Other important RNA molecules, such as microRNAs and long non-coding RNAs, can also play a role in regulating gene expression and thus form a pivotal fingerprint in tracking cellular changes that occur in cancer and common syndromes, such as intestinal brush border lactase deficiency [1, 2]. Despite the abundance of RNA molecules within cells, and their importance as prognostic tools in cancer research, the development of methods to detect mRNA molecules has been relatively delayed compared to the other biomarkers, namely DNA and proteins. The reason for this delay is primarily due to the instability of RNA molecules, which means that they can be degraded rapidly before detection. This has greatly impacted the discovery and monitoring of the aforementioned diseases by RNA levels [3-5].

Several techniques such as: Northern blotting, microarrays, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), digital or quantitative real time PCR (qPCR), and traditional RNA in situ hybridisation (ISH) were developed over the last five decades to measure RNA molecule levels. However, these technologies have limitations. For example, Northern blotting, and PCR based techniques require RNA extraction – RNA molecules might be lost during this process. Furthermore, Northern blotting and PCR techniques are unable to determine the localisation of gene expression within cells/tissue. Traditional RNA ISH, which uses digoxigenin (DIG) or radioactive probes, was developed to detect RNA molecules internally based on branched DNA (bDNA) method and principle [3,6]. However, a major limitation of traditional RNA ISH is that it cannot detect other than highly expressed genes e.g. *H19* (an imprinted maternally expressed transcript), because of the high degree of non-specific binding (lack of specificity) and resultant background noise (poor sensitivity) [3, 7]. Given these limitations, in 2012 RNAscope was introduced by Advanced Cell Diagnostics (ACD), Inc. as a novel improved technology of traditional RNA ISH [7, 8].

1.2 RNAscope technique

1.2.1 Underlying principle of the technology

Similar to traditional RNA ISH, RNAscope is based on the basic principle that RNA probes can be designed to detect a particular RNA of interest by hybridising to its complementary sequence inside the cell (the

cytoplasm in the case of mRNA molecules). In contrast to traditional ISH, where a single RNA sequence is conjugated with a label such as digoxigenin or a fluorophore, RNAscope uses a pair of 'Z' probes to detect the RNA of interest [9]. These 'Z' probes are comprised of three elements – the lower region that hybridises to RNA molecules, the spacer (linker) sequence that connects the lower region with the 'Z' probe tail, and the tail that binds to the pre-amplifier sequence (Figure 1A) [3]. Once the bottom of the double 'Z' probes (RNA specific sequence) bind to their target RNA sequence inside the cell, signal amplification is achieved through a series of sequential processes (Figure 1B) [3]. Firstly, the pre-amplifiers attach to their binding sites at the top of each double 'Z' pair. Secondly, multiple amplifier sequences bind subsequently via complementary base pairing to the pre-amplifier sequence. Finally, labelled probes, which can be either chromogenic or fluorescent, conjugate to their specific sites on the amplifier molecules.

The unique design of the 'Z' probes constitutes the main reason for the high specificity of RNAscope, which can reach 100% [3, 10]. The assay requires 'Z' probes to form a dimer on the target RNA sequence so the pre-amplifier can bind, and the amplification cascade can start. The features of the 'Z' probe design: (i) allows for single molecule detection, (ii) facilitates recognising very short molecules and thus partially degraded molecules and samples, and (iii) makes off-target binding very unlikely and thus suppresses background noise. The high sensitivity of RNAscope, which can also reach 100%, is due to the mechanism of the amplification process [3]. The unique process of signal amplification contributes significantly to the high sensitivity and specificity levels for RNAscope. Each RNA molecule should be hybridised to twenty 'Z' dimers (pre-amplifier). Each pre-amplifier in turn attaches to twenty amplifiers which can subsequently be attached by twenty labelled probes per amplifier. This process results in up to 8000 times signal amplification as 400 labelled probes will attach to each dimer.

1.2.2 Overall workflow

The RNAscope workflow starts with slide preparation, which should be performed according to the type of tissue being used: formalin fixed paraffin embedded (FFPE) tissues (most commonly), tissue microarrays (TMA), fresh frozen tissues, or fixed cells [10, 11]. Prepared slides then proceed through three key steps where the main principle of RNAscope is applied: permeabilization, hybridization and signal amplification. Moreover, these three key steps can be performed automatically as part of an automated RNAscope workflow [10]. The workflow process ends with the visualisation of results using a bright-field or fluorescent microscope (depending

on the probe type) and slides can be digitally scanned to facilitate quantification of the results, which can be performed either manually or by using a suitable computer software application [3, 12] (Figure 1C).

1.2.3 RNAscope controls

RNAscope quality is validated and assessed using positive and negative controls [10, 13]. The negative control probe utilises the bacterial gene *dapB* (dihydrodipicolinate B.subtilis reductase) to confirm the absence of background noise, as it is a gene which should not be present in any animal samples. On the other hand, a positive control is required to validate the detection of a signal resulting from expression of a gene that should be present in the tissue, such as a house-keeping gene. The positive control also acts as a measure of the tissue integrity – its failure to be detected would indicate degradation of RNA molecules. The most commonly used positive control is *PPIB* (peptidylprolyl isomerase B), which is employed for target genes that have moderate expression levels (10-30 copies per cell) [13, 14]. *Polr2A* (RNA polymerase II subunit A) is used for genes with low level of expression (3-15 copies per cell). *UBC* (Ubiquitin C) is generally used for highly expressed genes (> 20 copies per cell), but can also be utilised for target genes with moderate expression [14].

1.2.4 Analysis of RNAscope results

The analysis of RNAscope results involves quantification of the number of labelled dots within the tissue [8]. Each dot represents one RNA molecule, and thus the number of dots is indicative of the number of RNA molecules present – this is the critical factor to evaluate. However, it is noteworthy that sometimes, like in the case of highly expressed housekeeping genes, the dots can be found in clusters which makes them difficult to distinguish separately. It is also important to highlight that the intensity and size of each dot reflects the number of double Z probes (as opposed to the number of transcripts) which are bound to the target molecule and thus will vary.

Scoring of RNAscope staining can be done either manually or by using computer software [8]. For manual scoring, standards are suggested by the manufacturer, where several regions on the slide should be quantitated in order to obtain a comprehensive result. Several computer software programs have been developed to read, analyse, and quantify RNAscope results such as Halo, QuPath, and Aperio software [15]. Using these programs requires scanning the whole slide comprehensively – images of the slide should be taken from at least three directions [8]. Halo is one of the gold standard programs that analyse ISH image results quantitatively.

It is an adaptable platform with various advantages, including: scalability, powerful analytic capabilities, and high processing speed, which are applicable for both TMA and FFPE slides [16, 17].

1.2.5 RNAscope features

It is noteworthy that RNAscope can be used to assess heterogeneity between cells as it detects individual transcripts in a single cell. Furthermore, multiplex analysis can be performed to detect several genes within a single slide by using multiple probes with multiple channels – unique probes can be designed for each specific target, with each probe having a specific colour [18]. Additionally, RNAscope can be used in conjunction with immunohistochemistry (using either chromogenic or fluorescent detection) on the same tissue section as opposed to adjacent sections to allow the simultaneous detection of RNA and protein within the same tissue section [19]. Figure S1 (A-D) illustrates key features of RNAscope that are represented by results analysis.

1.3 Current applications of RNAscope

Since its introduction in 2012, RNAscope has been used widely to study gene expression in the context of basic scientific research studies in diverse areas such as neuroscience, stem cells, and developmental biology [12, 20, 21]. Furthermore, RNAscope has been applied in retrospective studies of clinical samples from non-infectious (e.g. cancer) and infectious (e.g. human papillomavirus (HPV) and, of great current interest, COVID-19) disease states [12, 20-23]. Interestingly, Neau *et al,* 2019 [24] have also highlighted the potential for integrating RNAscope to the biopharma field as a follow-up after gene expression analysis in the 3D culture process of organotypic cells, which is a vital tool in toxicology assessments and drug discovery. They also indicated that RNAscope has a promising future to be part of a comprehensive approach in tandem with omics data to assess histopathological samples. It is important to highlight that RNAscope can also be used in combination with other techniques such as microarray and immunohistochemistry (IHC), in a complementary way to confirm the results through producing data that cannot be achieved by IHC and microarray [10, 25].

1.4 Diagnosis of disease based on analysis of gene expression

1.4.1 'Gold standard' techniques and their limitations

Monitoring and analysis of gene expression is essential for the clinical diagnosis of a variety of diseases including cancers, infections (viruses, bacteria), cardiovascular, inflammatory, neurological, and many more [2]. The currently used 'gold standard' methods for analysis of gene expression in clinical diagnostics have some challenges and drawbacks that need solutions and improvements. As mentioned earlier, traditional RNA ISH, which has been developed over the last forty years, has several limitations and is a time-consuming method that requires complex procedures [3]. While conventional PCR and PCR-based methods provide robust information regarding absolute gene expression with a high degree of specificity and sensitivity, they do not provide spatial information of gene expression within cells or tissues [21].

IHC detects protein content via the use of specific antibodies that recognise a protein of interest [26, 27]. IHC is considered to be a cost-effective and robust method and is commonly used in the diagnostic setting, for example to detect E6/E7 proteins in HPV driven cancers [28]. However, suitable antibodies for a protein of interest may not be commercially available and thus, would have to be developed. Antibodies are available for only 25% of the human proteome, and new antibody development takes between six months to more than one year. This prolonged process impacts the detection of novel biomarkers or genetic signatures that are discovered as part of the clinical research process [26]. Another key limitation for IHC relates to antibody standardization. Promising antibodies which are used in the research field are not standardized, which can result in variability in the observed staining between studies [27]. Although antibodies which are used for IHC in the clinical diagnostic setting are standardized, the process to achieve standardization is lengthy, time consuming and expensive. A final limitation for IHC staining is that it lacks sensitivity for the target protein of interest in some cases, making it difficult to evaluate cases at the borderline of the limit of detection [29, 30].

1.4.2 Potential advantages and disadvantages of using RNAscope in clinical diagnostic testing

RNAscope has many advantages that indicate its potential to be utilised in clinical diagnostics [7, 21]: in principle, (i) it can detect the expression of any gene from any genome, which makes it suitable for diagnosis of infectious diseases [30]; (ii) it can detect low levels of gene expression that exist inherently or due to tissue degradation as seen in clinical FFPE material [12]; (iii) it has very high sensitivity and specificity [30]; (iv) it provides both a quantitative level of gene expression and spatial information regarding where the gene is expressed

within the tissue [7]; (v) RNAscope can be used in combination with IHC in the same tissue section to detect both RNA's and proteins that are implicated in disease diagnosis [19]; (vi) the design and preparation of the required probes is relatively short at just three days to two weeks (Personal communication from Andreas Rossbach, Advanced Cell Diagnostics (ACD)); (vii) it can be performed in multiplex format to detect more than one gene (up to three genes) simultaneously within the same tissue [7], which in turn allows for different cell populations to be distinguished from each other [3]; and (viii) RNAscope can be carried out in a high throughput manner and is suitable for automation [7]. Collectively, the incorporation of RNAscope into the clinical diagnostic field could have a significant impact on the diagnosis of many diseases.

The main drawbacks of the RNAscope technique, however, are the cost and workflow duration. There are large variations in costs for RNAscope among countries but using the UK as an example, the cost is estimated at around £65 per slide in comparison to £11 for IHC per slide (Personal communication from CRUK Beatson Institute histology department). Also, according to University College of London (UCL), $48 is required for the analytical process per each RNAscope stained slide [31]. Additionally, using the Leica Bond Rx autostainer, the required time for a full run of RNAscope (30 slides) is around 9 hours as compared to approximately 3 hours for IHC. However, the manual protocol for RNAscope could be completed in a working day, taking approximately 6.5-7 hours as compared to about 3 hours for IHC (Personal communication from CRUK Beatson Institute histology department).

1.5 Research objective, and strategy

The primary objective of this systematic review is to assess RNAscope performance compared to the existing 'gold standard' technologies that are currently used for gene expression analysis (qPCR, qRT-PCR, RNA ISH, and IHC) and gene detection (DNA ISH). To that end, this systematic review will focus on literature that has compared RNAscope to one or more of the existing techniques in human samples. The main criteria for comparison between RNAscope and the available technologies will encompass the concordance rate, sensitivity, and specificity. In addition, this systematic review will discuss some aspects and steps that will be required to validate RNAscope for clinical diagnostic testing.

2. Methodology

Two reviewers (SA. and SS.) independently conducted the database searching, screening, and data extraction from the identified articles in accordance with Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines [32].

2.1 Database search

Initially, a population, intervention, comparable group, outcome, and study design (PICOS) strategy was planned and followed to produce a robust research question and develop the required criteria for inclusion of eligible studies [33]. The population (P) in our study refers to human samples. The goal of this review was to measure multiple outcomes that related to diagnostic accuracy (with no condition to have all of them in the same article), thus the outcome was excluded. The included domains were the intervention group (I) indicated by the tested technique (RNAscope), the comparable group (C) that refers to the various techniques that are being used as the 'gold standard' techniques (immunohistochemistry (IHC), Northern blotting, microarray, qPCR, qRT-PCR and DNA ISH), and the study design (S) to include only articles that were primary scientific experimental research studies.

Literature searching for this systematic review was performed in four databases: CINAHL (Cumulative Index to Nursing & Allied Health) (EBSCOhost), Web of Science, Medline, and Embase. The last conducted search was on the 24th of November, 2020. The search was conducted in all the databases using the same key terms, Boolean operators, and strategy, however, different wildcards, truncation, phrase searching, and adjacency tools were used in accordance with guidelines for each database (Tables S1-S4). As RNAscope is a very recent method, it did not apply under any specific MeSH (Medical Subject Headings) term under RNAscope and RNA ISH terms.

2.2 Literature screening

The first stage of assessing the articles available in databases was screening for the search terms in the title, abstract, and keywords. The exclusion criteria were as follows (i) studies published before 2012, (ii) studies published in languages other than English, (iii) the study objective was not to compare RNAscope to other technique/s, (iv) the title and abstract do not include any of the included methods, (v) the intended meaning of

RNA ISH was traditional RNA ISH rather than RNAscope, and (vi) the samples used were non-human samples. Inclusion criteria were to retain any study that was conducted after 2012 that had full-text access which compared RNAscope to one or more of the 'gold standard' techniques, and the study aim was to evaluate the comparison of the methods.

2.3 Data extraction and quality assessment

The results were extracted from the selected articles and tabulated under five main sections in accordance with the Cochrane Collaboration template [34] – general information, comparable techniques, methods, results, and conclusions. The extracted data was collated and re-tabulated into three main tables: concordance rate and measures of diagnostic accuracy (sensitivity, and specificity) of RNAscope (Table 1); the types of cancer tissues and genes which were studied (Table S6); and general information and details relating to the RNAscope methodology employed (Table S7).

The aim of this systematic review is to assess a new diagnostic technique (RNAscope). Therefore, QUADAS-2 (Quality Assessment of Diagnostic Accuracy Studies) was selected to assess the quality and applicability of the included studies [35]. This method appraises the quality of four key criteria in the studies: sample selection; index test; reference standard; and flow and timing, by using several guiding questions in each domain. A modified version of the standard QUADAS-2 template (Supplementary Document 1) was used in this systematic review to evaluate each study. Specific cut off points were defined to assess the total risk of bias as follows: (i) the total risk was considered 'low' when three or four out of four domains were low risk, (ii) a rating of 'some concerns' was given when two of the domains have a high or unclear risk of bias and (iii) overall risk was considered 'high' when three or four out of four domains had a high or unclear risk of bias.

2.4 Data synthesis

Data was synthesized and the results were reviewed and analysed in a qualitative and narrative manner to answer the main research question. Meta-analysis could not be performed because of: (i) the heterogeneity of samples, comparable groups, obtained outcomes and (ii) insufficient data being reported in included articles with respect to important parameters required for meta-analysis, including sensitivity, specificity, false positives, false negatives and concordance rate.

3. Results

3.1 Article selection (flow-chart results)

Using four different databases, a group of 16,457 articles was identified by the literature searching strategy. A total of 4884 articles remained after the exclusion of published articles before 2012. After duplicates were removed, 4011 titles were screened to end up with 581 articles. Based on the eligibility criteria, a further 490 articles were excluded after abstracts were screened. Finally, full-text screening led to the selection of twenty-seven articles to be included in this systematic review (Figure 2A).

3.2 Quality assessment – Risk of Bias (RoB)

The quality of each of the included studies was assessed using QUADAS-2 tool. Ten out of the twenty-seven articles (37%) were classified to have some concerns in the risk of bias (RoB) assessment (Table S5). The majority of high and unclear risk of bias results were concentrated in the sample selection criteria and reference standard domains, whereas the index test and flow and timing domains had the lowest RoB assessment (Figure 2B).

3.3 Study characteristics

3.3.1 Techniques

Table 1 and Figure 3 represent the main data extracted from the selected articles. The publication dates extended from 2013 to 2020. All the selected articles compared RNAscope technique to one or more of the current gold standard techniques. The main 'gold standard' method compared to RNAScope was IHC, as evident by eleven articles (40.7%) that compared it to IHC only, and fifteen articles (#1,3,4,8-10,12,13,24,25,27) (55.6%) that compared it to IHC and other techniques simultaneously (#2,5-7,11,14-22,26).

In addition to RNAscope, two gold standard techniques were used in seven studies; where five studies compared RNAscope to both IHC and fluorescent ISH (FISH) or DNA ISH (#6,14,18,20,22), and the other two studies compared RNAscope to both IHC and qPCR (#2,17). Five studies compared RNAscope to three techniques; IHC, FISH, and qPCR (#5,7,15,16,19), whereas one study compared RNAscope to four techniques,

including IHC, qPCR, Chromogenic ISH (CISH), and dual ISH (#26). The remaining article compared RNAscope with duplicated techniques other than IHC, namely real time DNA and qRT-PCR (#23) (Figure 3A).

3.3.2 Tissues

Twenty-six out of twenty-seven of the included studies (96.3%) used cancer tissue samples, out of which head and neck squamous cell carcinoma (HNSCC) was the most studied cancer type (in eight articles) (29.6%) (#5-8,17,19,21,23). Lung cancer was the second most common cancer type studied (in five articles) (18.5%) (#4,9,10,18,24) (Figure 3B-3C). Only one article studied CMV and EBV viruses obtained from inflammatory cases (#20). Although RNAscope is used to measure all types of RNA molecules, only mRNA was measured in all of the included articles.

3.3.3 Genes studied and biomarkers

The genes which were included in the selected articles were next scrutinised. Interestingly, ten articles (37%) focused on *E6/ E7* transcripts of *HPV* in *HPV* driven cancers (HNSCC, squamous cell carcinoma (SCC), and anogenital neoplasia) (#5-8,15-17,19,21,23). Several markers related to immune checkpoints, including: programmed cell death ligand (*PD-L1*) (#4,9,10,25), and *B7-H3 and B7-H4* (#13), were also assessed. Another three papers investigated prognostic receptors in breast cancer, including epidermal growth factor receptor, *HER-2* (#11,26) and the nuclear hormone receptor, *Erα* (Estrogen receptor α) (#27). Two studies evaluated glycoproteins with prognostic values, like Podoplanin *(PDPN)* (#3) and glypican3 (*GPC3*) and glutamine synthetase (*GS*) (#1). Other genes studies included: the tumour suppressor genes, *PTEN* (Phosphatase and tensin homolog) (#2) and *SPARC* (Secreted protein acidic and rich in cysteine) (#12). The remaining four articles evaluated *MDM2*, anaplastic lymphoma kinase (*ALK*), *MYB, Napsin A* (the aspartic protease), and *TTF1* (Thyroid Transcription Factor 1) genes, respectively (#14,18,22,24) (Figure 3C, Table S6).

3.3.4 Methods of staining and quantifications

Automated RNAscope was used in eleven studies (40.7%) (#4,8-10,13,14,16,20,22,23,26). RNAscope was conducted in a mixed way (manual and automated) in one article (3.7%) (#6). Full manual RNAscope was used in the remaining fifteen articles (55.6%) (#1-3,5,7,11,12,15,17-19,21,24,25,27). The method for quantification was automated in four studies (#2,10,21,26), mixed manual and automated in one study (#25), and manual in the remaining twenty-one studies. The method of quantification was unclear in one study (#7). Although only five studies used the automated scoring system, none of them used the same software. SpotStudio from ACD

was the software of choice for Bingham *et al,* 2015 (#2); QuPath was used by Humphries *et al,* 2018 (#10); Rooper *et al,* 2016 (#21) carried the analysis out using ViewRNA program; Tretiakova *et al,* 2018 (#25) used web-based Spectrum Plus digital slide manager; and custom software was used by Wang *et al,* 2013 (#26).

3.3.5 RNAscope controls

In terms of positive controls which were employed, four studies (14.8%) used *UBC* (#2,16,17,23), while fourteen studies (51.9%) used *PPIB* (#1,5,8,10,12,13,15,18-20,22,24,27). One study (3.7%) used *Polr2A* in tandem with *UBC* as the positive controls (#26). Three out of the 8 remaining articles (11.1%) used appropriate controls without mentioning the probe which was used (#6,9,21). However, the last five articles (#3,4,11,14,25) (18.5%) did not mention the use of any positive control in their studies. With regards to negative controls, *dapB* was used in sixteen (#1,2,5,8,10,12,13,15-17,19,20,23,24,26,27) (59.3%) of the included articles. Appropriate controls were used in three (#6,9,21) (11.1%) of the eleven remaining articles, but without the mention of the specific probes. The last eight articles (#3,4,7,11,14,18,22,25) (29.6%) did not mention the use of negative control in their studies. Chromogenic probes were used in twenty-five articles (92.6%) (#1-25), while two articles (7.4%) used the florescent probes (#26,27) (Table S7).

3.4 Concordance rate (CR)

The CR was stated and extracted from sixteen of the included articles (59.3%) (#4-8,11-13,15,17,18,20,23,25-27). Fourteen papers (#4-8,11-13,17,18,20,25-27) estimated CR of RNAscope with IHC, out of which, IHC was co-compared to another technique in six (#7,11,17,18,20,26) of the fourteen studies. Five articles (#7,11,15,17,26) calculated CR against PCR. Although one study evaluated several techniques compared to RNAscope, the CR of RNAscope was reported against PCR method only (#15). Four of the sixteen studies (#7,11,18,26) calculated CR of RNAscope against DNA ISH or FISH among other techniques used in these studies. A high level of variability in CR (58.7% - 95.3%) was reported in the studies that compared RNAscope to IHC (Figure 4A). However, studies that compared RNAscope to qPCR and DNA ISH demonstrated relatively close CR, within a range of 89% - 97.3% and 82% - 100%, respectively (Figure 4B-4C). The CR between RNAscope and qRT-PCR was reported only in one study as 78% (#23). Silver-enhanced in situ hybridization (SISH) technique also showed high concordance (90.5%) with RNAscope (#11) (Figure 4D). One study reported low CR between RNAscope and IHC, but no numerical data was provided (#2). Similarly, two articles reported high CR between RNAscope and IHC without providing percentages (#2, 19).

The authors of the included studies where the CR between RNAscope and IHC was relatively and unexpectedly low (#4,13,20,25) provided several possible reasons to explain these results. For example, Bingham *et al*, 2015 referred to the existence of different mechanisms of gene regulation at both the transcriptional (mRNA) and posttranscriptional (protein) levels. Similarly, Kim *et al*, 2018 referred to the possibility of inadequate translation of the required gene into protein, which might be due to impaired posttranscriptional processing of the mRNA transcript or the repression of translation initiation. In contrast, Tretiakova *et al*, 2018 criticised the IHC technique, arguing that antibodies directed against PD-L1 require more standardization and validation.

3.5 The accuracy of RNAscope (Sensitivity, and Specificity)

Although determining the sensitivity and specificity of RNAscope was not considered as a primary aim within the included studies, fifteen studies (55.6%) estimated both sensitivity and specificity ratios (#1,5,6,9,13-15,17-19,21-24,26), and one study (3.7%) estimated only the sensitivity value of RNAscope (#16). Overall, the reported sensitivity and specificity results were relatively high in all of these studies. The sensitivity values ranged between 48% to100%, with a median value of 94.3%. Whereas the specificity ranged between 75% to 100%, with a median value of 93% (Figure 5).

Eight out of these fifteen articles also provided estimates of the sensitivity and specificity of the other included techniques (IHC, DNA ISH, etc.) (Table S8). The sensitivity of the RNAscope was reportedly similar or superior to the other techniques in six articles (#IHC: 1,14,16; DNA ISH: 5,15,16,19; others: 5,16,19). However, IHC sensitivity outperformed RNAscope in three articles (#5,9,22) (Figure 5A). The specificity of RNAscope exceeds the specificity of the other techniques in five articles (#IHC: 1,5,14,22; DNA ISH: 5; others: 5,19). On the other hand, the specificity ratios for IHC and DNA ISH were higher than RNAscope in two articles; (#9) and (#19), respectively (Figure 5B).

3.6 Results and clinical outcomes/ diagnosis

The clinical utility of RNAscope was highlighted in some of the selected articles for its potential in providing accurate diagnosis and prognosis for certain conditions such as cancer and infections, particularly those which are caused by viruses. Nine articles (33.3%) recorded the relationship between RNAscope results and clinical outcomes (#1,4-6,9,12,13,23,25). Bakheet *et al,* 2020 (#1) suggested that using RNAscope will improve

the pathological and differential diagnoses of hepatocellular carcinoma at early stages. Coppock *et al,* 2018 (#4) did not report any significant difference between the ability of RNAscope and IHC to predict patient survival rate (IHC predicted an average of 5.3 months; RNAscope predicted an average of 5.2 months). Craig *et al,* 2020 (#5) demonstrated that the use of RNAscope in detecting HPV is superior to IHC as it is predicted to reduce the false positive/ negative cases by almost half; although there was no difference between RNAscope and DNA ISH with respect to ability to detect the virus. In contrast, Daneshpajouhnejad *et al,* 2020 (#6) nominated RNAscope over DNA ISH for the diagnostic process as it provides an interface with easier features for interpretation. Gafeer *et al,* 2018 (#9) recommended using RNAscope in conjunction with IHC in the diagnostic process as it provides more accurate information to assist in determining the patients' eligibility to receive immunotherapy. Kim *et al,* 2018 and Kim *et al,* 2017 (#12,13) did not report a significant difference between RNAscope and IHC in predicting the disease recurrence rate as this was found to be almost the same for positive cases using both techniques. Similarly, Schache *et al*, 2013 (#23) found qRT-PCR and RNAscope to be equally good with regards to predicting and discriminating patient survival rate as both techniques predicted the same survival rate for the patients. In contrast, the ability of RNAscope to predict patient survival rate in comparison to IHC was found to be unclear in the study by Tretiakova *et al,* 2018 (#25).

3.7 RNAscope advantages and disadvantages

The included articles contained consideration of advantages and disadvantages for using RNAscope compared to the other gold standard techniques, and these are summarised in Table 2. The main advantage was that RNAscope was considered as an innovative technique demonstrating a high degree of accuracy and ability to detect any gene in a short time frame. However, the major disadvantage was cost because RNAscope is an expensive technique compared to IHC.

4. Discussion

According to the authors' knowledge, this is the first systematic review to be conducted on the RNAscope technique comparing its use against gold-standard diagnostic methods. RNAscope is a relatively new technique and while there are more than 500 papers that cited RNAscope [20], only twenty-seven articles were identified which focused on evaluating RNAscope in comparison to current gold standard techniques that measure gene expression levels or detect genes.

4.1 Quality assessment

The quality assessment findings demonstrated that all the included articles had an overall score of low or some concerns for RoB, demonstrating that this systematic review provides high quality data. However, it should be noted that personal bias can still be introduced [58].

The most affected domains with respect to RoB were sample selection and reference standard. The high scores in these domains were attributed to ambiguity related to sample selection (random vs consecutive) because most of the samples were retrieved from biobanks or storage. Biobank samples might not be selected randomly or consecutively in the first place, which allows for potential bias. Articles also scored high or unclear RoB for the reference standard domain because RNAscope is still considered as a recent technique, for which a reference standard is yet to be developed. Each of the included articles used a different gold standard technique, but with variations in the procedures (reagents, conditions, and scoring methods) that were employed. For this technique to be adopted into the clinical diagnostic field, a reliable and accurate reference standard for RNAscope should be designed so that the test accuracy can be normalised. Furthermore, producing a reliable and accurate reference standard requires careful consideration of the staining and quantification of elements of RNAscope, a process that involves two separate procedures that should be evaluated separately.

Although the possibility of bias can be reduced when automated systems are employed in either the staining or the scoring stages of RNAscope assays [59], less than half of the included studies in this systematic review utilized automated systems. This might be because the goal of the included studies was to validate RNAscope, for which a manual method was used in order to fully assess all stages of the technique. However, during the full-text screening process for this systematic review, many of the recently conducted articles that have used RNAscope as an experimental method in scientific research were noted to use either partial or fully

automated systems. In the manual scoring method, no special training is required to produce reliable results [7]. However, manual scoring is a time-consuming method and at least two pathologists are needed validate the results [13, 25]. Automatic scoring methods are recommended over manual scoring, although the produced results will be robust either way if the proper guidelines are followed.

The quality of RNAscope was assessed through most of the included articles using positive and negative controls. Throughout this review, fourteen studies used *PPIB* as a positive control, indicating that target mRNAs being studied were products of low and moderately expressed genes. Being able to accurately detect and monitor small changes in gene expression, including genes which are low in abundance, is critical with respect to the diagnosis and management of cancer and certain infectious diseases [2]. The high degree of specificity and sensitivity that is offered by RNAscope is highly advantageous in this regard. Furthermore, the relatively high CR for RNAscope with IHC for genes that are expressed at low levels including HER-2 gene emphasises the effectiveness of RNAscope for detecting such genes.

4.2 Study characteristics

Cancer development is caused by changes in gene expression that lead to uncontrolled and inappropriate cell growth [60]. It is not surprising, therefore, that almost all of the included articles focused on cancer [61]. A variety of cancer types were covered in the included studies – the majority of articles concentrated on HNSCC, followed by the most common cancers worldwide according to the world health organization (WHO) (2020 record) namely lung and breast cancers [62].

The E6/E7 transcripts of HPV have a critical role in the development of cancer as they can transform cells and they have the ability to deregulate important tumour suppressor genes, including p53, Rb, and others, which leads to uncontrolled cell proliferation and induction of oncogenesis [63]. E6/E7 was identified as the most investigated target in the selected articles in this systematic review, as evident in 10 articles (#5-8,15-17,19,21,23) (37%). Although HPV is established to contribute to the development of cervical cancer (in 90% of cases) [64], through this systematic review, HPV was tested in the context of cervical cancer in only one of the included articles versus nine which were in the context of HNSCC. In light of this observation, it is noteworthy that there is an increasing body of evidence for HPV infection as a risk factor for HNSCC [65].

One of the main hallmarks of cancer is the evasion of the immune system [66]. Cancer can bypass the immune system by modulating key immune markers that are vital in suppressing the host anti-tumour response, in which effector T cells play a vital role [67]. Programmed cell death protein (PD-1) and B7-H3 are key examples of such markers and it is of great interest that they were studied in some of the included articles (#4,9,10,13,25). The presence of PD-1 and B7-H3 are co-inhibitory to effector T cell function – upon the interaction of effector T cells with tumour cells, the presence of PD-1 and B7-H3 on the surface of tumour cells results in inactivation, tolerance and anergy of the effector T cells, leading to uncontrolled cell growth in the cancer. The identification of such markers has allowed for the development of targeted therapies against these checkpoints, and immune checkpoint inhibition treatment has proven to be successful in various cancers [61]. However, treatment success is highly dependent upon investigation of the expression of these markers in patients to determine those who are most likely to respond to immune checkpoint inhibition. RNAscope could be a highly valuable tool in this diagnostic process.

4.3 Concordance rate (CR)

The CRs between RNAscope and IHC varied considerably across the included articles. The main reason for lack of concordance was attributed to the difference between RNA and protein content (#4,13,20,25). Furthermore, Yu *et al,* 2017 [45] highlighted that protein content, but not RNA, might change due to gene mutations. In their study of sixty-two genes in eight cancer types, Jia and Zhongming, 2017 [68] drew attention to post-translational processes such as phosphorylation and glycosylation, which can affect protein, but not RNA expression. However, the fact that proteins are produced from raw RNA molecules (with coding and non-coding sequences) that could be translated differently into several proteins [69] explains the relatively low CR between RNAscope and IHC.

Interestingly, the CR between RNAscope and qPCR or qRT-PCR (presented in 6 studies) was relatively high (89%-97.3%) compared to IHC (calculated in fourteen studies). This is likely because RNAscope, qPCR, and qRT-PCR measure the same molecule, RNA. Further studies are required to fully evaluate RNAscope CR with IHC, qPCR, and qRT-PCR, but interestingly, it was proposed by Bingham *et al,* 2015 [29] that combining RNAscope with IHC methods might produce more robust results than using either technique alone, resulting in greater accuracy. This notion is supported by Kang *et al,* 2013 [70]. DNA ISH has also demonstrated a strong CR

(> 82%) with RNAscope, which is not unexpected as RNA is directly transcribed from DNA and only a small amount of data might be lost [69].

4.4 The accuracy of RNAscope (Sensitivity, and specificity)

Regardless of the sample size, most sensitivity and specificity ratios reported in this systematic review were relatively high, which is in keeping with other reports in the literature [3, 10] and suggests that RNAscope is a robust technique that would be suitable for the diagnostic field. Three articles that compared RNAscope to IHC reported relatively low sensitivity levels, but only Kim *et al,* 2018 [43] indicated the reasons for the observed low sensitivity of RNAscope (48.4%) compared to IHC (51.6%) might be due to: (i) the increased translation rate of mRNA to protein molecules, (ii) a decrease in the elimination of activator proteins in the tissue, which function to increase gene transcription [71] and (iii) small sample size introducing bias in the results.

The studies which reported a large difference in sensitivity of RNAscope compared to IHC (#1,14) highlight the importance of using techniques that measure the same molecule (RNA) as a reference standard to validate RNAscope as opposed to techniques which measure a different molecule, for instance IHC which measures protein. Since 2009, several techniques have been developed to measure RNA molecules. Single cell RNA sequencing (sscRNA-seq) is the most notable of these – it has many of the advantages of RNAscope and is approved for clinical diagnostics [72]. However, none of the studies identified in the research process of this systematic review included sscRNA-seq as a comparative technique to RNAscope. Also, gene expression profiling (GEP) method that utilises either microarray or sequencing technologies is used to show the pattern of the expressed genes by measuring mRNA levels [73, 74]. In 2013, Handorf *et al* [75] compared the accuracy of IHC to GEP method and it was around 71% compared to 91%, respectively.

Although the reported sensitivity and specificity values for RNAscope are high in included articles in this systematic review, there is insufficient data in the included studies to fully evaluate the suitability of RNAscope as an independent test – further appraisal of accuracy values [sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV)] for RNAscope as part of prospective studies is clearly needed before it could be adopted as a stand-alone test in the clinical diagnostic field. An important consideration relates to the level of expression of the gene being detected e.g HPV E6/E7 are highly expressed genes and so it is not surprising that RNAscope recorded high specificity results for their detection [76]. In order to fully evaluate

whether RNAscope has superior specificity compared to the gold standard techniques, more studies on RNAscope focusing on genes with low expression levels should be conducted. Furthermore, it would be important to evaluate RNAscope effectiveness within low-risk populations as all the included articles in the systematic review relate to high-risk populations and sub-populations within these.

Comparing techniques that measure the same variable is recommended as part of the validation process for adoption of new techniques into the clinical diagnostic setting [77]. Furthermore, it is important to consider other variables in addition to CR and accuracy parameters as part of the validation process of a new technique, including benefit to the patient and test management.

4.5 Results and clinical outcomes

Within the included articles, ten studies reported the correlation between RNAscope results and clinical outcomes. Five studies (#4,12,13,18,23) out of the ten did not report any difference on the recurrence or survival rates. Two of the remaining three articles (#5,9) recommend using RNAscope with IHC for better diagnosis and prediction of patients' eligibility for treatment with immunotherapy. One study (#25) indicated that there was no clear correlation between using RNAscope and the survival rate. The last two studies (#6,26) nominated a preference for using RNAscope over the other techniques for better diagnosis and evaluation of prognosis. This was indicated by Wang and his colleagues [78] in 2014 where they have measured HPV E6/E7 gene expression levels using RNAscope to predict the status of oropharyngeal SCC. They found that RNAscope was a good predictive method. On the basis of data and evidence in the articles included in this systematic review, the effectiveness of RNAscope as a prognostic tool remains to be fully determined and further studies are required to confirm this relationship.

4.6 Regulations

When introducing a new technique to the clinical diagnostic system, a long process of several steps should be followed to validate the technique analytically and clinically in addition to consideration of the ethical, legal, and social implications of the test [77]. As part of the validation process, it should be considered as a quantitative test, and thus its trueness and robustness should be evaluated. RNAscope trueness should be measured

correctly to evaluate the predicted bias either systematically or proportionally, and trueness should be adjusted using the appropriate correction factors. The robustness, which is represented by the precision of the quantitative test, should also be validated within either the run, the laboratory itself, or among several laboratories [79]. Adhering to these steps is critical to validate RNAscope and thus intercalate it into the clinical diagnostic field.

4.7 Costs

Cost-effectiveness is one of the most important aspects to consider with regards to implementing a new technique for the diagnostic system. Given the high cost of RNAscope, an incremental cost approach should be adopted as part of RNAscope cost-effectiveness analysis (CEA), which considers how cost relates to the potential outcome [80]. The incremental approach would consider how the direct costs [reagents (probes, staining kits, etc.) and equipment (e.g. specific machines and required software programs to analyse and quantify the data)] and other related costs [staff (including their training and recruitment) and building costs] would change if RNAscope were to be adopted. In the case of adopting RNAscope as a new technique, the outcome of any CEA study would recommend whether the benefits of implementing RNAscope would outweigh the costs of its introduction.

4.8 Limitations of this systematic review

This systematic review only included twenty-seven studies that met the critera. The included studies did not all have the same objective. For example, some studies compared RNAscope to IHC alone, while some compared it to more than one different technique. Also, some articles involved more than one research question, which introduces bias during the data extraction process. Not all the included articles reported important parameters relating to test accuracy (sensitivity, specificity, PPV, NPV, concordance), which prevented meta-analysis and statistics from being performed and so it is difficult to draw firm conclusions. Ideally, the grey literature should be searched to avoid any source of bias in the results synthesis process. Furthermore, the bibliographies of relevant reviews should be searched manually. Due to time constraints, neither type of these searches was performed.

Another limitation of the current systematic review relates to publication bias, which might have been introduced throughout the search strategy, data extraction, quality assessment, or data analysis procedures [81]. With respect to the selection criteria, included studies were restricted to full text articles that were available in the

English language, which might have excluded articles in languages other than English as well as unpublished data in relevant topics. However, several procedures were followed throughout the multiple steps which were performed in generating this systematic review to avoid bias. For instance, the literature search was conducted on a large scale by using four different databases. Additionally, the screeding and selection criteria were comprehensive as they included all possible groups. Furthermore, the quality of the included articles was assessed using the QUADAS-2 tool to ensure the high quality and accuracy of the included articles. One limitation relating to the quality assessment tool (QUADAS-2) that might have introduced some bias is that RNAscope is still a new technique, and thus, no reference standard was available at the time this systematic review was conducted. For this reason, a new criterion was produced in the QUADAS-2 tool to avoid having many articles with a high RoB in the second domain (index test).

5. Conclusion

In conclusion, this systematic review is considered the first conducted review in this field. All the included articles focused on validating RNAscope against (an)other technique(s). This review demonstrated that RNAscope is a promising and accurate technique, as exemplified by high degrees of sensitivity and specificity. The authors of this study would recommend the adoption of RNAscope as a complementary method in the clinical field to confirm unclear results from other techniques, as it offers advantages and solutions for the current challenges of gold standard techniques. However, further studies comparing RNAscope to scRNA-seq and qRT-PCR techniques are needed to fully assess the effectiveness of RNAscope so it can be incorporated as a fully independent method to diagnose gene expression disorders. To validate the high specificity of RNAscope, more studies concentrating on low expressed genes should be conducted. Furthermore, diagnostic accuracy values of the technique should be evaluated in prospective studies to obtain firmer conclusions regarding sensitivity, and specificity ratios. Finally, many validation steps (analytically, clinically, and logistically) are needed to assess RNAscope more broadly and profoundly, and thus to apply it to the clinical diagnostic field.

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7. Figure legends

Figure 1 – The elements of 'Z' probes. (A) The constituents of 'Z' probe dimers are: 1. the lower region that comprises 18 to 25 bases per each 'Z' probe; 2. linker sequence. 3. the tail that comprises 14 bases per each 'Z' probe. This figure panel was created with Powerpoint using data from Wang et al (2012) [3]. (**B)** The sequential steps of RNAscope involve: 1. binding of double 'Z' probes to a complementary sequence; 2. attachment of pre-amplifier to double 'Z' pair tail; 3. binding of amplification molecules (amplifiers) to pre-amplifier; 4. attachment of the labelled probes to their specific sites on the amplifiers. This figure panel was created with Powerpoint using data from Erben and Buonanno (2019) [8] and Wang *et al.* (2012) [3]. **(C)** The presented flow-chart illustrates the RNAscope workflow process and highlights which parts can be automated (steps 2, 3, and 4). This figure panel was created with Powerpoint using data from [3, 10-12].

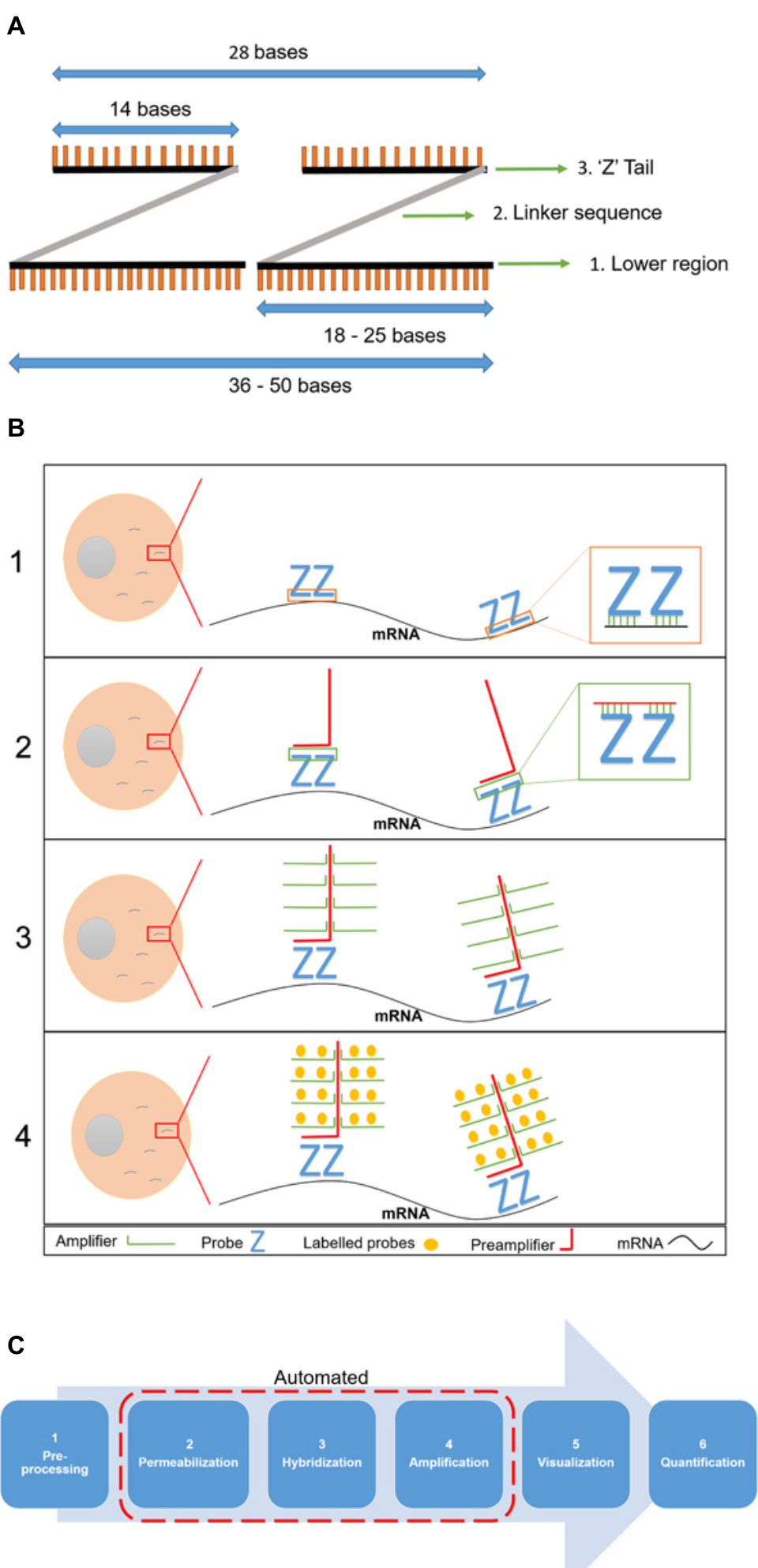
Figure 2 – PRISMA flow diagram and assessment of risk of bias. (A) The presented flow chart outlines and summarizes the main research steps which were taken in the sequential selection of the articles included in the systematic review, including an explanation of the exclusion criteria for each step. Adapted from PRISMA [32]. **(B)** The presented bar chart illustrates the percentage of studies for each RoB level within each domain for the included studies as determined using QUADAS-2 tool. The green colour represents a low risk, the yellow colour represents an unclear risk, and the red colour represents a high risk.

Figure 3 – Evaluation of Study Characteristics out of the twenty-seven articles. The presented pie charts illustrate: **(A)** the percentage of studies using specified current gold standard techniques that were compared to RNAscope; **(B)** the percentages of studies using samples from specified types of cancer in the included articles. **(C)** the percentages of studies using specified markers within the included articles.

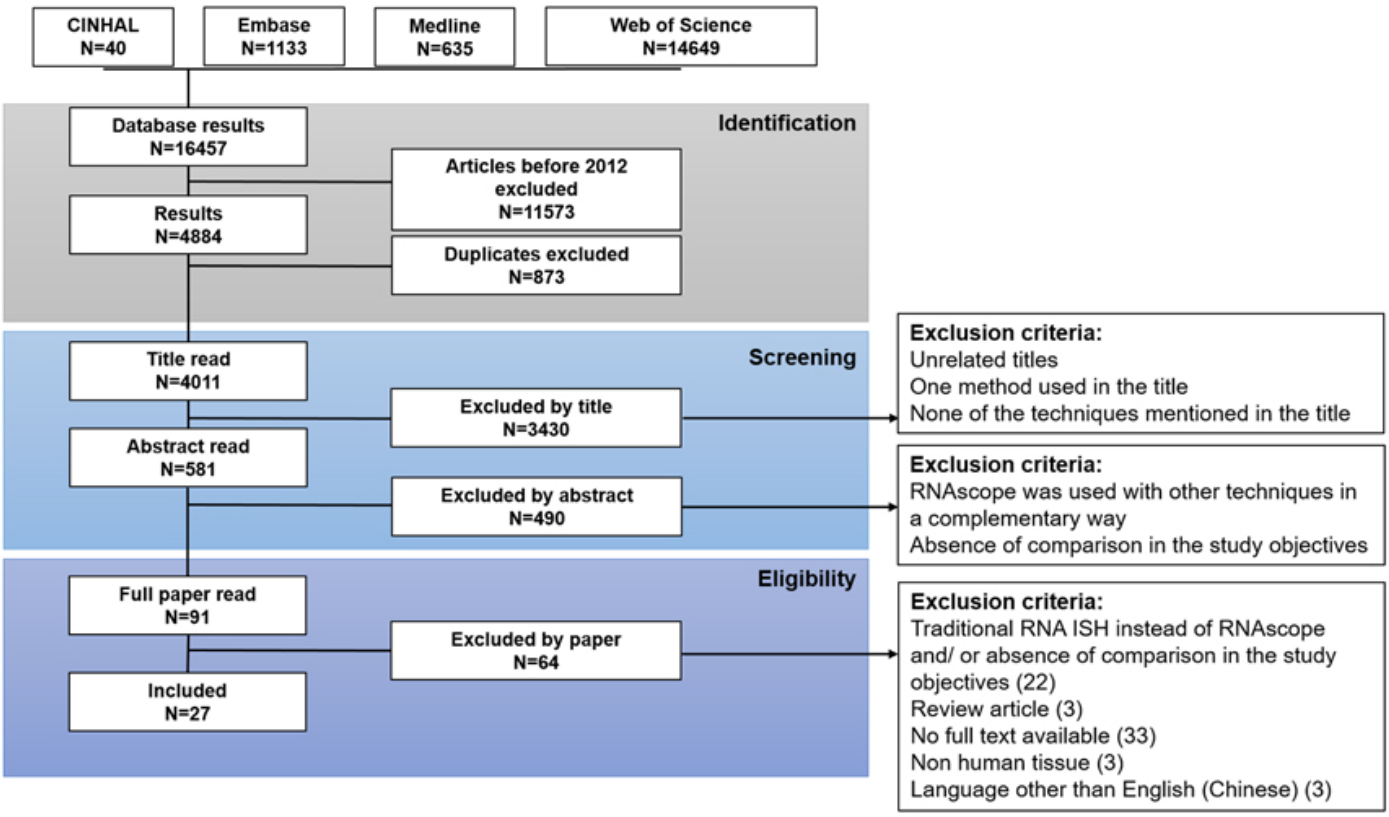
Abbreviations: Adenoid cystic carcinoma (AdCC), Breast cancer (BC), Cytomegalovirus (CMV), Epstein-Barr virus (EBV), ERα: Estrogen receptor α, head and neck squamous cell carcinoma (HNSCC), Hepatocellular carcinoma (HCC), HER-2 : human epidermal growth factor receptor 2, NSCLC: Non-small-cell lung carcinoma, Podoplanin (PDPN), PTEN: Phosphatase and tensin homolog, PT: phyllodes tumours, PD-L1: Programmed death-ligand 1, SCC: squamous cell carcinoma, SPARC: Secreted protein acidic and rich in cysteine, TTF1: Thyroid Transcription Factor 1.

Figure 4 – Evaluation of the concordance rate between the results of RNAscope and IHC, qPCR, DNA ISH. The presented bar charts illustrate the CR results from: **(A)** 14 studies that compared RNAscope to IHC; **(B)** 5 studies that compared RNAscope to qPCR; **(C)** 4 studies that compared RNAscope to DNA ISH; and **(D)** 2 studies that compared RNAscope to other studies like qRT-PCR and SISH.

Figure 5 – The sensitivity and specificity ratios of RNAscope vs. other techniques. The presented bar charts illustrate: **(A)** sensitivity ratios for RNAscope (13 studies) and other techniques whose values were co-reported in the same studies. Top graph – IHC (co-reported in 6 studies); Middle graph – DNA ISH (co-reported in 4 studies); Lower Graph – DNA PCR (co-reported in 3 studies) and **(B)** specificity ratios for RNAscope (11 studies) and other techniques whose values were co-reported in the same studies. Top graph – IHC (co-reported in 5 studies); Middle graph – DNA ISH (co-reported in 2 studies); Lower Graph – DNA PCR (co-reported in 2 studies**).**


A
28 bases
14 bases
3. 'Z' Tail
2. Linker sequence
1. Lower region
18 - 25 bases
36 - 50 bases
B
1
2
3
4
mRNA
Amplifier
Probe
Labelled probes
Preamplifier
mRNA
C
Automated
1 Pre-processing
2 Permeabilization
3 Hybridization
4 Amplification
5 Visualization
6 Quantification

A


CINHAL
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Embase
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Medline
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Web of Science
N=14649
Identification
Database results
N=16457
Articles before 2012
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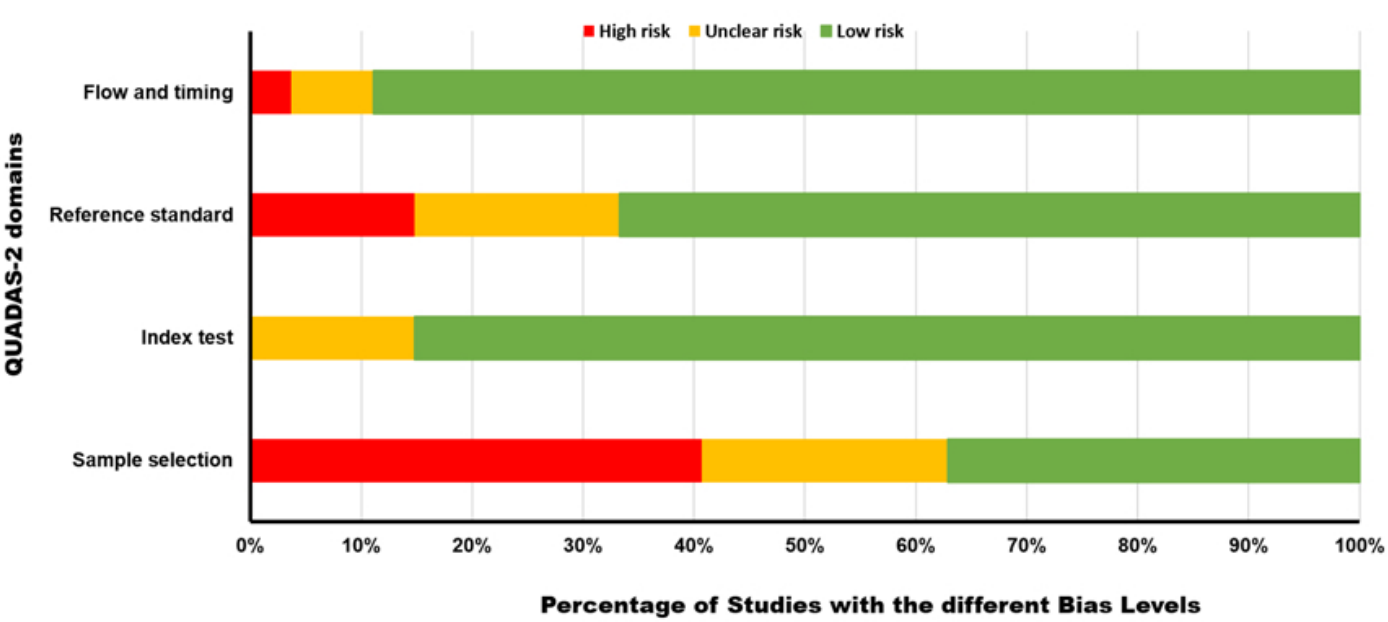
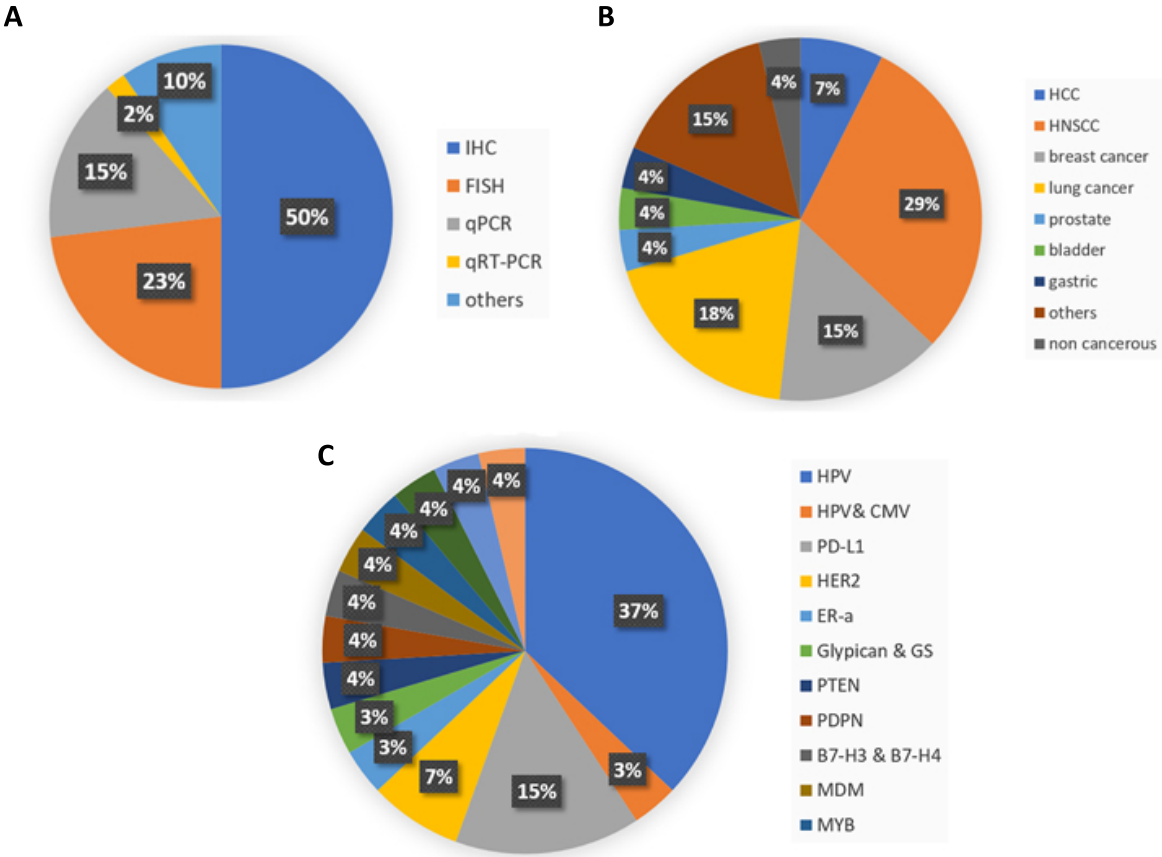
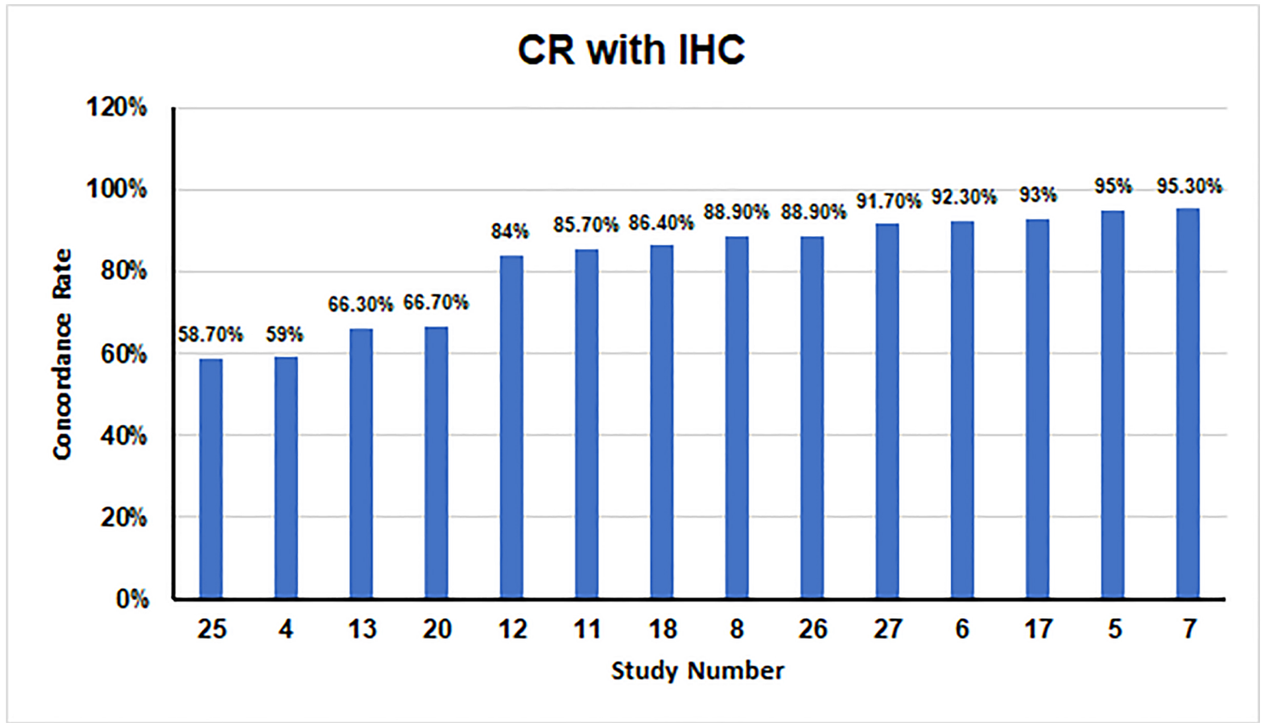
N=11573
Results
N=4884
Duplicates excluded
N=873
Screening
Title read
N=4011
Excluded by title
N=3430
Abstract read
N=581
Excluded by abstract
N=490
Eligibility
Full paper read
N=91
Excluded by paper
N=64
Included
N=27
Exclusion criteria:
Unrelated titles
One method used in the title
None of the techniques mentioned in the title
Exclusion criteria:
RNAscope was used with other techniques in a complementary way
Absence of comparison in the study objectives
Exclusion criteria:
Traditional RNA ISH instead of RNAscope and/ or absence of comparison in the study objectives (22)
Review article (3)
No full text available (33)
Non human tissue (3)
Language other than English (Chinese) (3)

B

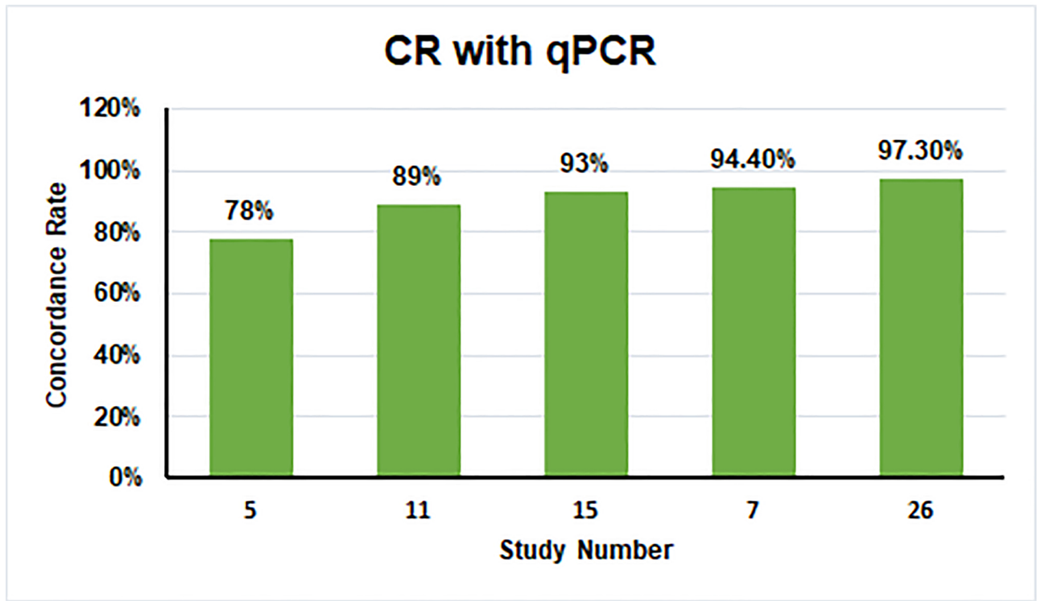

High risk
Unclear risk
Low risk
QUADAS-2 domains
Flow and timing
Reference standard
Index test
Sample selection
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Percentage of Studies with the different Bias Levels


A
50%
23%
15%
2%
10%
IHC
FISH
qPCR
qRT-PCR
others
B
29%
15%
18%
4%
4%
4%
15%
4%
7%
HCC
HNSCC
breast cancer
lung cancer
prostate
bladder
gastric
others
non cancerous
C
37%
3%
15%
7%
3%
3%
4%
4%
4%
4%
4%
4%
4%
4%
4%
HPV
HPV& CMV
PD-L1
HER2
ER-a
Glypican & GS
PTEN
PDPN
B7-H3 & B7-H4
MDM
MYB

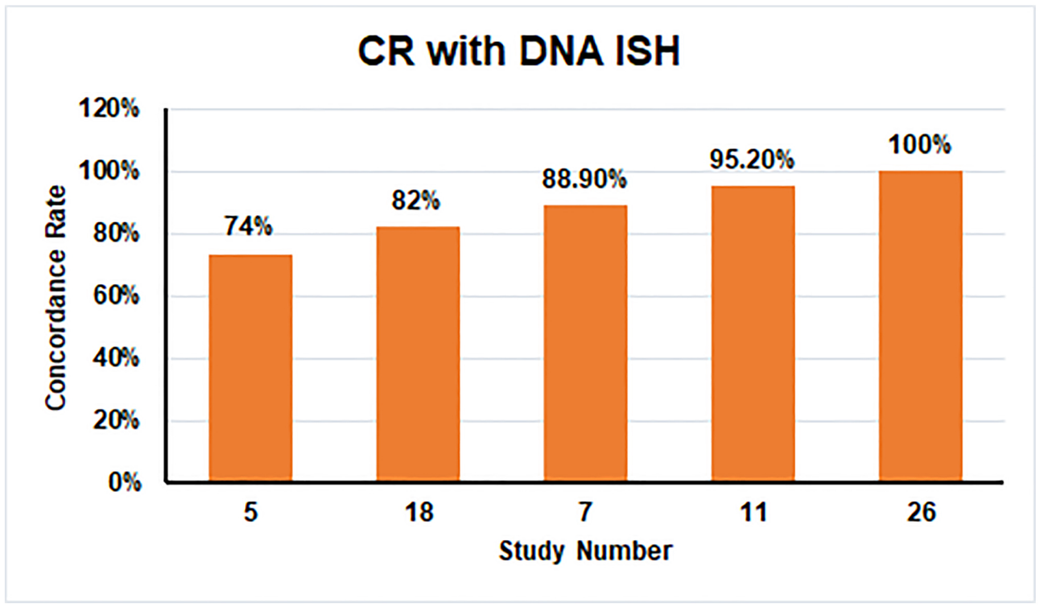
A


CR with IHC
Concordance Rate
120%
100%
80%
60%
40%
20%
0%
58.70%
59%
66.30%
66.70%
84%
85.70%
86.40%
88.90%
88.90%
91.70%
92.30%
93%
95%
95.30%
25
4
13
20
12
11
18
8
26
27
6
17
5
7
Study Number

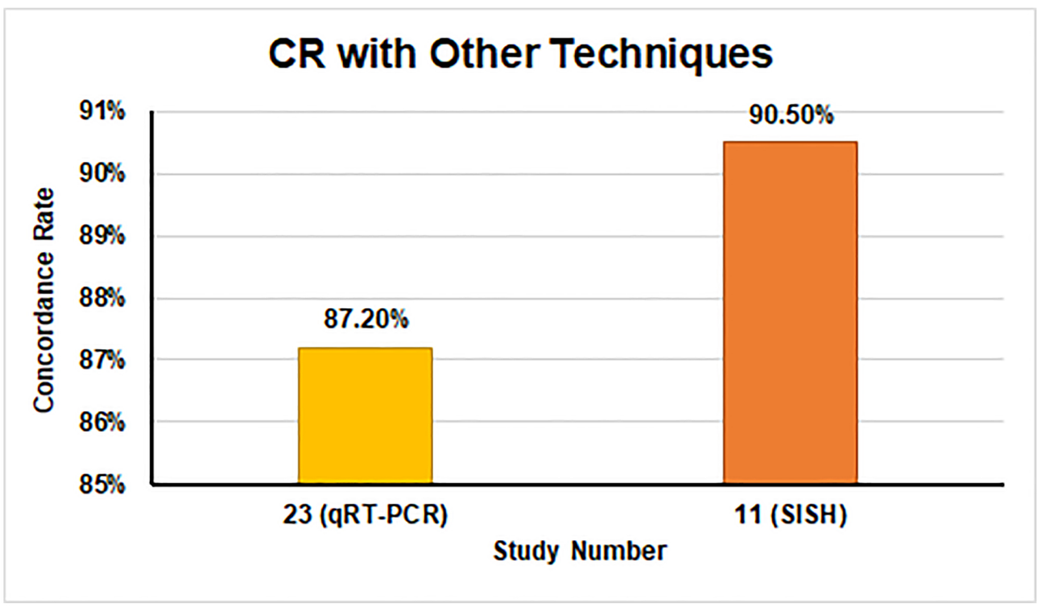
B


CR with qPCR
Concordance Rate
120%
100%
80%
60%
40%
20%
0%
78%
89%
93%
94.40%
97.30%
5
11
15
7
26
Study Number

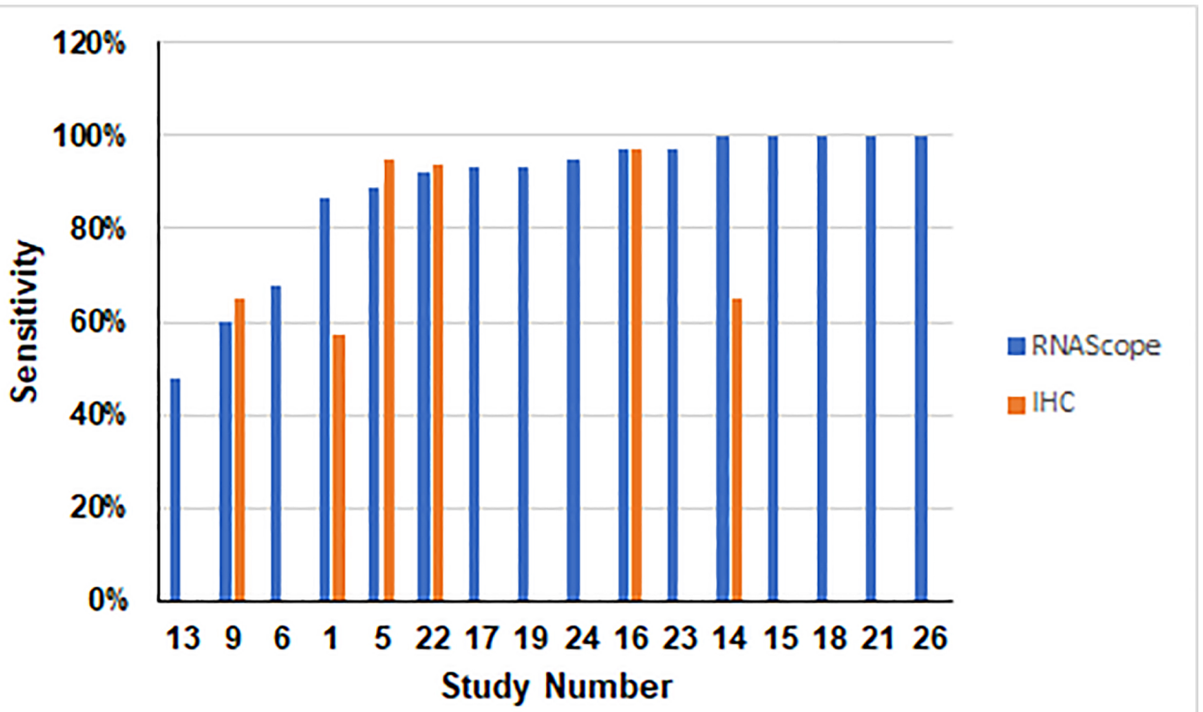
C


CR with DNA ISH
Concordance Rate
120%
100%
80%
60%
40%
20%
0%
74%
82%
88.90%
95.20%
100%
5
18
7
11
26
Study Number

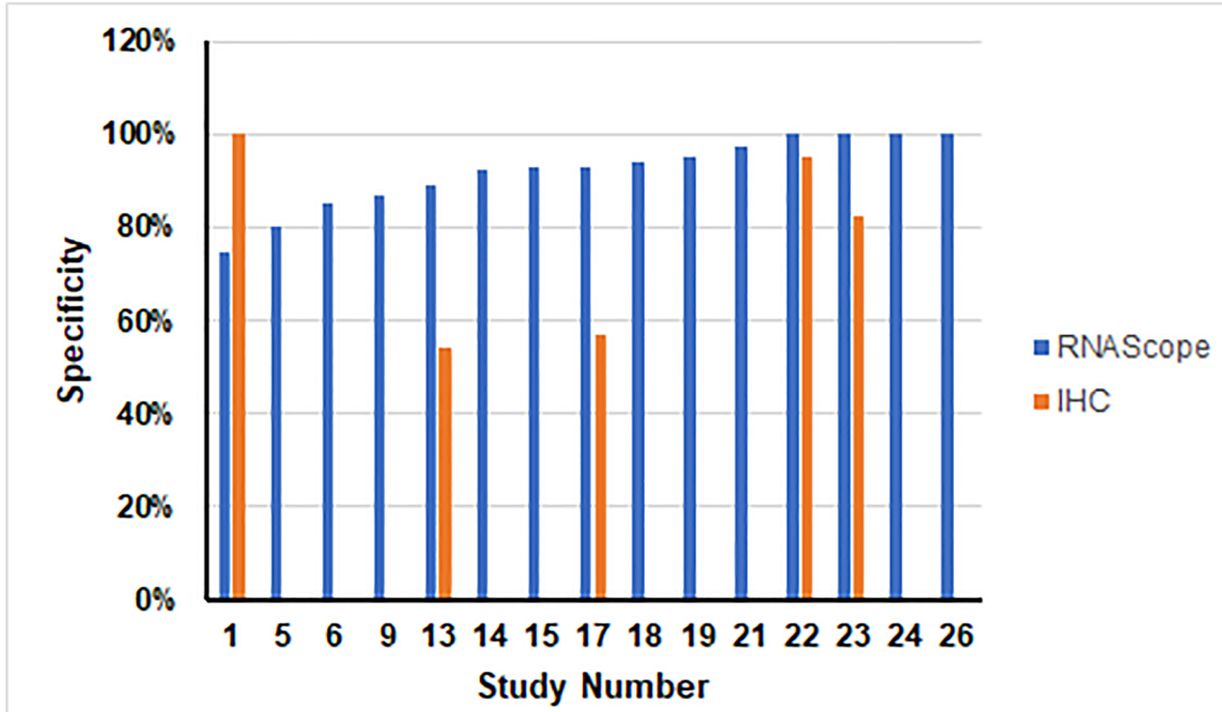
D


CR with Other Techniques
Concordance Rate
91%
90%
89%
88%
87%
86%
85%
87.20%
90.50%
23 (qRT-PCR)
11 (SISH)
Study Number

A


120%
100%
80%
60%
40%
20%
0%
Sensitivity
13 9 6 1 5 22 17 19 24 16 23 14 15 18 21 26
Study Number
RNAScope
IHC

B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Specificity
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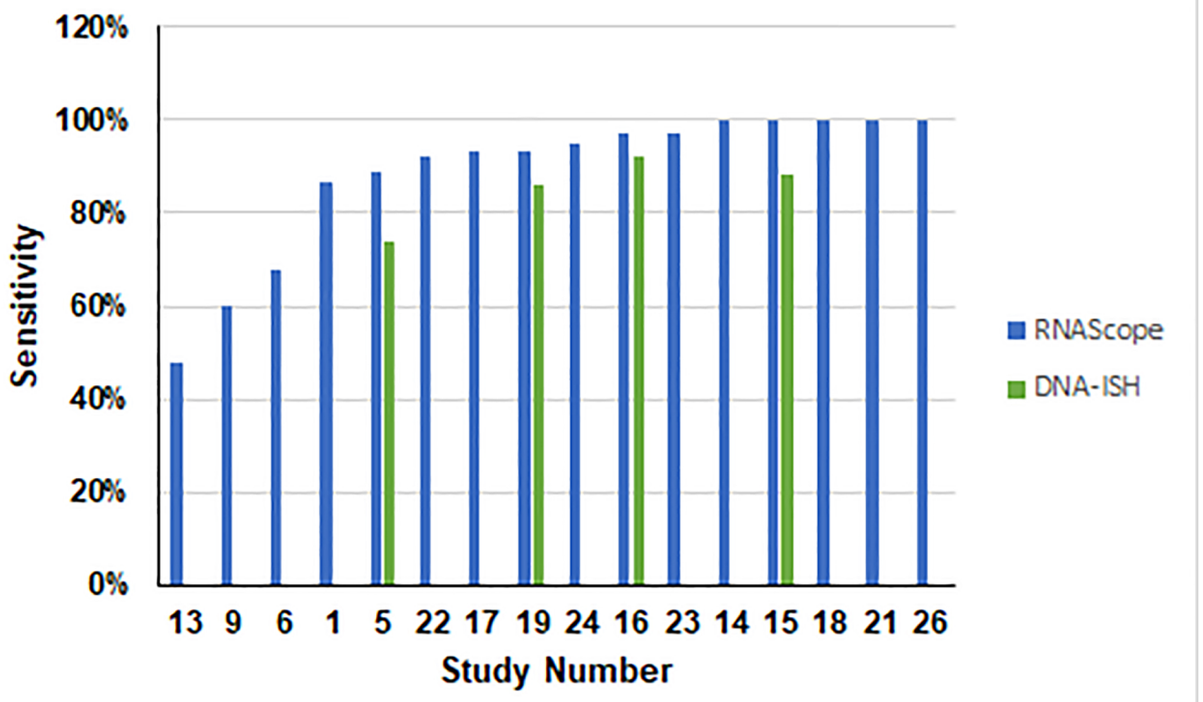
Study Number
RNAScope
IHC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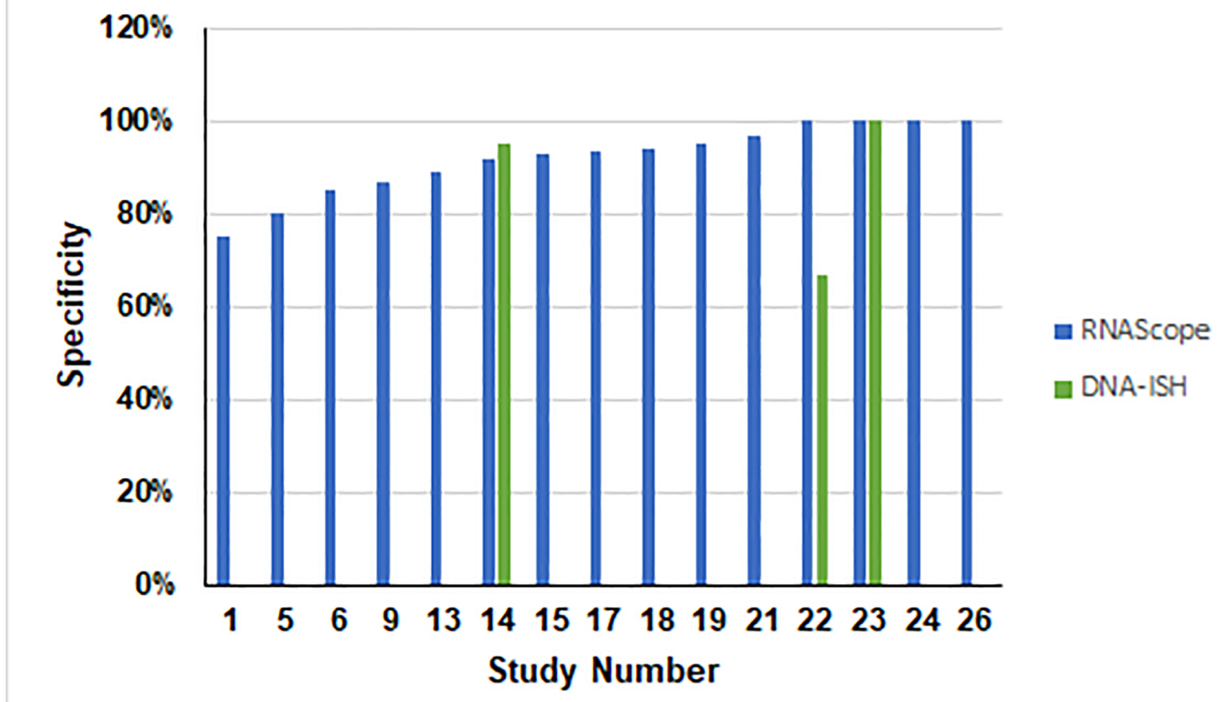
Sensitivity
13 9 6 1 5 22 17 19 24 16 23 14 15 18 21 26
Study Number
RNAScope
DNA-ISH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Specificity
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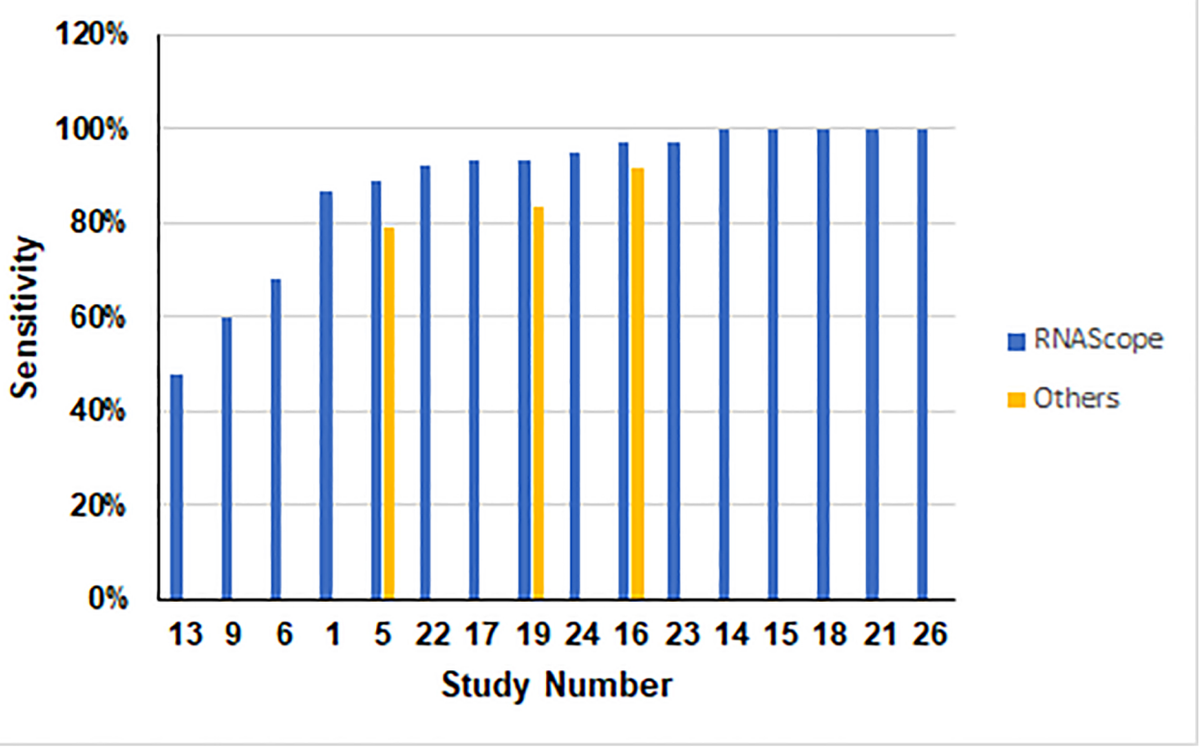
Study Number
RNAScope
DNA-ISH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20%
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Sensitivity
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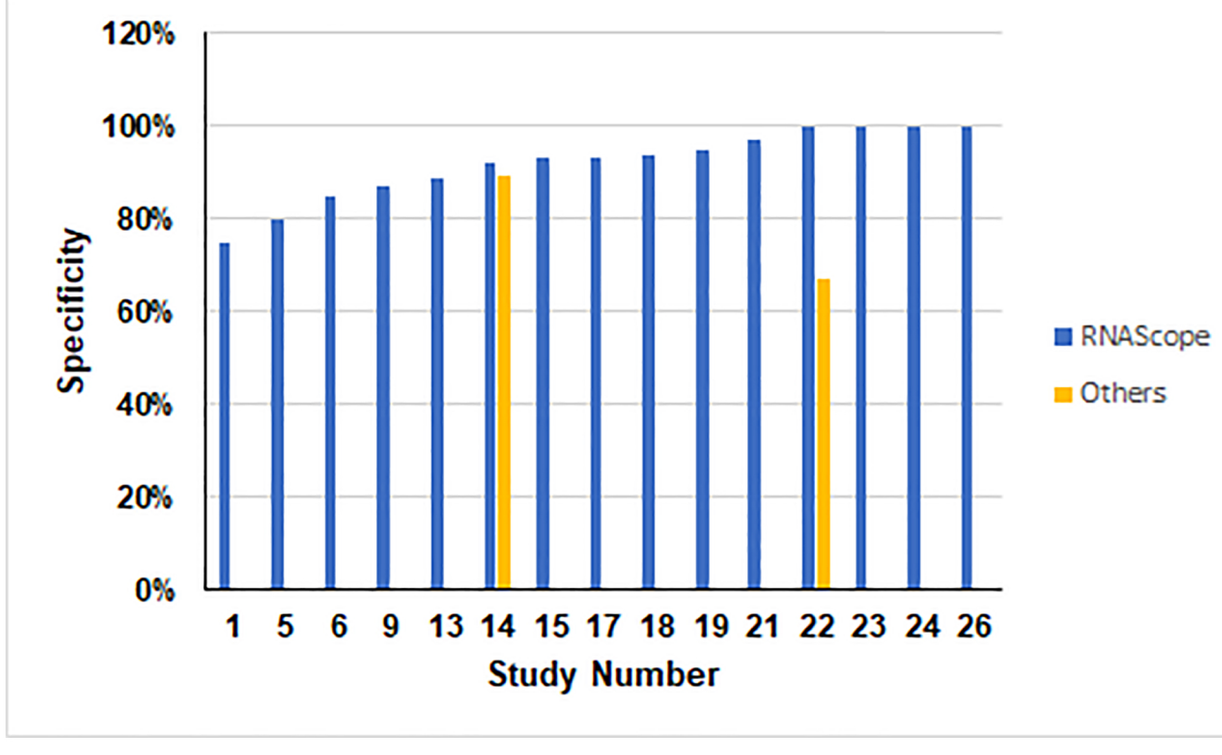
Study Number
RNAScope
Others


120%
100%
80%
60%
40%
20%
0%
Specificity
1 5 6 9 13 14 15 17 18 19 21 22 23 24 26
Study Number
RNAScope
Others

Manuscript Tables

Table 1 – Concordance rate and accuracy values of RNAscope.

The study numbers (Study No.) in this table will be used throughout the SR to refer to these articles.

Abbreviations: (FISH): fluorescent in-situ hybridization; (IHC): immunohistochemistry; (NPV): negative predictive value; (PCR): polymerase chain reaction; (PPV): positive predictive value: (SISH): Silver-enhanced in situ hybridization

Study No.	Ref. No.	Author and Publication year	Study type	Sample size	Concordance Rate (CR)				Sensitivity	Specificity	PPV	NPV
					IHC	PCR	FISH	Others				
1	36	Bakheet et al, 2020	Retrospective	194					86.4%	93.2%		
2	27	Bingham *et al,* 2015	Retrospective	127								
3	37	Cioca et al, 2017	Retrospective	20								
4	38	Coppock *et al,* 2018	Retrospective	112	59%							
5	26	Craig et al, 2020	Retrospective	221	95%				89%	100%		
6	46	Daneshpajouhnejad et al, 2020	Retrospective	65	92.30%				67.7%	80%		
7	47	Dreyer *et al*, 2013	Retrospective	64	95.30%	94.40% (of +ve)	88.90% (of +ve)					
8	39	Drumheller *et al*, 2019	Retrospective	27	88.90%							
9	40	Gafeer *et al*, 2018	Retrospective	20					60%	75%	92%	27%
10	41	Humphries *et al,* 2018	Retrospective	813								
11	48	Kim *et al*, 2013	Retrospective	211	85.70%	89%	95.20%	90.50% (SISH*)				
12	42	Kim *et al,* 2017	Retrospective	82	84%							
13	43	Kim *et al,* 2018	Retrospective	101	66.30%				48.4%	97.30%		
14	49	Kulkarni et al, 2019	Retrospective	109					100%	100%		
15	50	Mendez-Pena *et al*, 2017	Retrospective	57		93%			100%	87%	87%	100%
16	51	Mills *et al*, 2017	Retrospective	127					97%			

17	24	Mirghani *et al,* 2015	Retrospective	50	90%	93%			93%	94%	96%	
18	52	Nakajima *et al*, 2017	Retrospective	555	91%		81.8%		100%	100%		
19	53	Randen-Brady et al, 2019	Retrospective	357	82% Highly concordant				93.40%	92.4%	95.5%	89%
20	54	Roe *et al*, 2017	Retrospective	55	66.70%			90.3% (traditional ISH)			EBV*: 86.4% CMV*: 100%	EBV: 100% CMV: 50%
21	55	Rooper et al, 2016	Retrospective	82					100%	100%		
22	56	Rooper et al., 2020	Retrospective	320					92%	89%		
23	57	Schache *et al,* 2013	Retrospective	79				87.20% (qRT-PCR)	97%	93%	91%	98%
24	44	Shi *et al,* 2015	Retrospective	380					95%	85%		
25	25	Tretiakova *et al,* 2018	Retrospective	112	58.70%							
26	28	Wang *et al,* 2013	Retrospective	163	88.90%	97.30%	100%		100%	95.20%		
27	45	Yu *et al,* 2017	Retrospective	72	91.70%							

Cytomegalovirus (CMV), Epstein-Barr virus (EBV)

Table 2 – Advantages and disadvantages of RNAscope technique.

Factor	Study No.
Advantages	
Identify gene expression at a single-cell level within a morphological context	13, 18
Does not depend on antibodies	13
Allows the detection of mRNA as a single gene copy	12, 20
High analytical accuracy, sensitivity and specificity	1, 4, 7-9, 11,15, 17, 19-24
More reliable than IHC	3
Suppress background noise and produce better resolution than IHC	8, 15, 17, 20, 27
Reduce the risk of false positive results	17
Its results are easy to interpret	5, 6, 8, 15, 17, 21
It is a robust and quantitative technique	11, 16, 27
It can detect tissue heterogeneity and partially degraded RNA	2, 27
Quick to perform	9, 11, 18
It can be performed automatically and manually and saves time	1, 14
Disadvantages	
It is not suitable to discriminate between viral RNA transcripts and viral DNA.	7
The stain will not take place well if the samples are with poor fixation quality and the cost is much higher compared to IHC	11
In Cervical Intraepithelial Neoplasia (CIN) cases, the negativity of RNAscope does not guarantee the absence of HR-HPV	16
RNAscope was less specific differentiating AdCC from high grade basaloid sinonasal tumors.	22
In the automated system, some areas in the slides need manual selection during the scoring process	26

Supplementary: Evaluation of the suitability of RNAscope as a technique to measure gene expression in clinical diagnostics: a systematic review

Running Heading: Evaluation of RNAscope technique for gene expression in clinical diagnostics: A systematic search and review.

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Author Affiliations:

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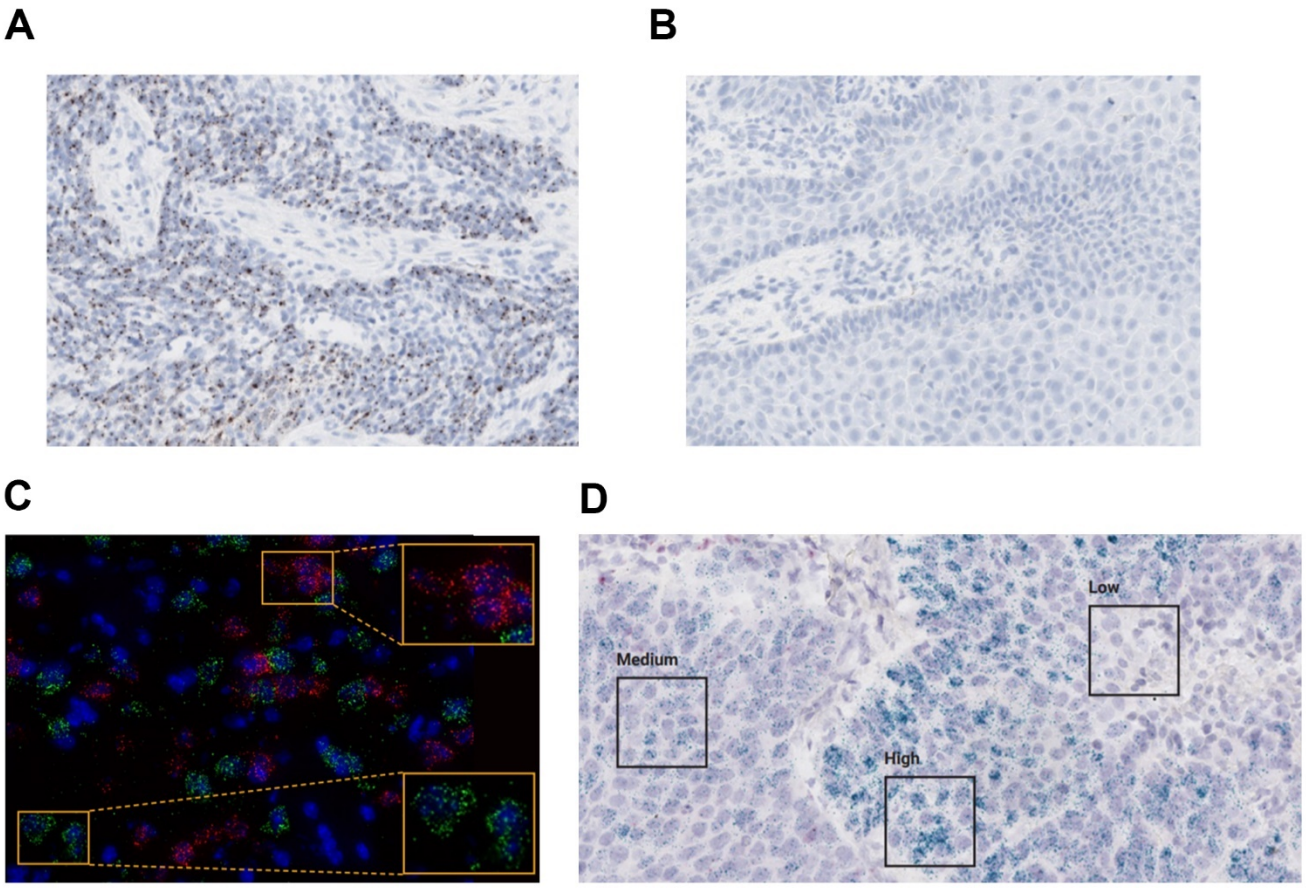
Supplementary Figures

Figure S1



Supplementary Figure Legends

Figure S1 – Example results using RNAscope probes for genes under investigation. (A) Positive sample for HPV (Human Papillomavirus) infection in human samples (condyloma tissue) from RNAscope chromogenic analysis. **(B)** Negative sample for HPV infection in human samples (head tumour sites) from RNAscope chromogenic analysis. **(C)** Fluorescent image where 2 different genes were stained using RNAscope in the same slide: *Drd1* (dopamine receptor D1) gene is represented by the red colour, while *Drd2* (dopamine receptor D2) gene is represented by the green colour. (**D)** RNAscope chromogenic image that shows several levels of gene expression (heterogeneity) across the tissue section. Figure reproduced from [82].

Supplementary Tables

Table S1 – Search strategy in Embase database.

Embase comprises literature from 1947-present and is updated daily. The last search was performed on 24th of November 2020.

No.	Search title	Search results
1	(rna?scope or rna-scope or rna?ish or rna-ish or rna in situ hybridization).ti,ab,kw.	2754
2	microarray.ti,ab,kw.	136332
3	(north blot or north-blot or northern blotting or north?blot).ti,ab,kw.	6446
4	(quantitative reverse transcription polymerase chain reaction or qRT-PCR or qRT?PCR or quantitative RT-PCR or quantitative RT-PCR).ti,ab,kw.	89261
5	(digital RT-PCR or digital RT?PCR or digital reverse transcription polymerase chain reaction).ti,ab,kw.	55
6	Immunohistochemistry.ab,kw,ti.	308260
7	(RNA?seq or RNA-seq or rna sequencing).ab,kw,ti.	67653
8	(dna?seq or dna-seq or ngs or next generation sequencing).ab,kw,ti.	68938
9	(DNA?ISH or dna-ish or dna in situ hybridisation).ab,kw,ti	107
10	Gene expression.ab,kw,ti.	553114
11	2 or 3 or 4 or 5 or 6 or 7 or 8 or 9	630065
12	1 and 11	**1133**

Table S2 – Search strategy in Medline database

Ovid (Medline) and Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Daily and Versions 1946 to November 24, 2020. Last search was performed on 24th. November 2020

No.	Search title	Search results
1	(rna?scope or rna-scope or rna?ish or rna-ish or rna in situ hybridization).ti,ab,kw.	1763
2	microarray.ti,ab,kw.	90727
3	(north blot or north-blot or northern blotting or north?blot).ti,ab,kw	6095
4	(quantitative reverse transcription polymerase chain reaction or qRT-PCR or qRT?PCR or quantitative RT-PCR or quantitative RT-PCR).ti,ab,kw.	57962
5	(digital RT-PCR or digital RT?PCR or digital reverse transcription polymerase chain reaction).ti,ab,kw.	37
6	Immunohistochemistry.ab,kw,ti.	193620
7	(RNA?seq or RNA-seq or rna sequencing).ab,kw,ti.	44957
8	(dna?seq or dna-seq or ngs or next generation sequencing).ab,kw,ti.	39899
9	(DNA?ISH or dna-ish or dna in situ hybridisation).ab,kw,ti	69
10	2 or 3 or 4 or 5 or 6 or 7 or 8 or 9	405778
11	1 and 10	**635**
12	Limit 11 to (full text and yr=”2012 – 2021” and English)	**99**

Table S3 – Search strategy in Web of Science database

Last search was performed on 24th. November 2020

No.	Search title	Search results
1	(rna$scope or rna-scope or rna$ish or rna-ish or rna in situ hybridization).	32,196
2	Microarray.	143,110
3	(north blot or north-blot or northern blotting or north$blot).	38,345
4	(quantitative reverse transcription polymerase chain reaction or qRT-PCR or qRT$PCR or quantitative RT-PCR or quantitative RT-PCR).	78,802
5	(digital RT-PCR or digital RT$PCR or digital reverse transcription polymerase chain reaction).	520
6	Immunohistochemistry.	217,060
7	(RNA$seq or RNA-seq or rna sequencing).	257,116
8	(dna$seq or dna-seq or ngs or next generation sequencing).	55,464
9	(DNA$ISH or dna-ish or dna in situ hybridisation).	66
10	2 or 3 or 4 or 5 or 6 or 7 or 8 or 9	726,277
11	1 and 10	**14,694**
12	1 and 10 (2012-2021)	**3,510**

Table S4 – Search strategy in CINAHL database

All of the keywords were applied to the title, abstract, and subject. Last search 24th. November. 2020.

No.	Search title	Search results
1	(rna#scope or rna-scope or rna#ish or rna-ish or rna in situ hybridization).	45
2	Microarray.	8,923
3	(north blot or north-blot or northern blotting or north#blot).	516
4	(quantitative reverse transcription polymerase chain reaction or qRT-PCR or qRT#PCR or quantitative RT-PCR or quantitative RT-PCR).	4,895
5	(digital RT-PCR or digital RT#PCR or digital reverse transcription polymerase chain reaction).	9
6	Immunohistochemistry.	39,974
7	(RNA#seq or RNA-seq or rna sequencing).	2,464
8	(dna#seq or dna-seq or ngs or next generation sequencing).	4,504
9	(DNA#ISH or dna-ish or dna in situ hybridisation).	5
10	Gene expression	51,242
11	2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10	95,889
12	1 and 11	**40**

Table S5 – Summary of the quality assessment tool results (risk of bias levels).

No.	Author	Sample selection	Index test	Reference standard	Flow and timing	Overall score
1	Bakheet et al, 2020	High	Low	Low	Low	Low
2	Bingham *et al,* 2015	Unclear	Low	High	Low	Some concerns
3	Cioca et al, 2017	Some concerns	Some concerns	Low	Low	Some concerns
4	Coppock *et al,* 2018	Low	Low	Low	Low	Low
5	Craig et al, 2020	Some concerns	Low	Low	Low	Low
6	Daneshpajouhnejad et al, 2020	Low	Low	Low	Low	Low
7	Dreyer *et al*, 2013	Low	Some concerns	Low	High	Some concerns
8	Drumheller *et al*, 2019	High	Low	Some concerns	Low	Some concerns
9	Gafeer *et al*, 2018	Low	Low	Some concerns	Low	Low
10	Humphries *et al,* 2018	Unclear	Low	Low	Low	Low
11	Kim *et al*, 2013	Low	Some concerns	Some concerns	Low	Some concerns
12	Kim *et al,* 2017	High	Low	High	Low	some concerns
13	Kim *et al,* 2018	Unclear	Low	High	Low	Some concerns
14	Kulkarni et al, 2019	Low	Low	Low	Low	Low
15	Mendez-Pena *et al*, 2017	High	Low	Low	Low	Low
16	Mills *et al*, 2017	Low	Low	Some concerns	Low	Low
17	Mirghani *et al,* 2015	Low	Low	High	Low	Low
18	Nakajima *et al*, 2017	High	Low	Low	Low	Low
19	Randen-Brady et al, 2019	High	Low	Low	Low	Low
20	Roe *et al*, 2017	High	Low	Some concerns	Low	Some concerns
21	Rooper et al, 2016	Some concerns	Low	Low	Some concerns	Some concerns

22	Rooper et al., 2020	Low	Low	Low	Low	Low
23	Schache *et al,* 2013	High	Low	Low	Low	Low
24	Shi *et al,* 2015	High	Low	Low	Low	Low
25	Tretiakova *et al,* 2018	High	Low	Low	Low	Low
26	Wang *et al,* 2013	Low	low	Low	Unclear	Low
27	Yu *et al,* 2017	High	Unclear	Low	Low	Some concerns

Table S6 – The various types of cancers and genes that were studied through the included articles of this SR.

Abbreviations: (AdCC): adenoid cystic carcinoma; (BC): breast cancer; (ERα): estrogen receptor α; (HNSCC): head and neck squamous cell carcinoma; (HCC): hepatocellular carcinoma; (HER-2) : human epidermal growth factor receptor 2; (NSCLC): non-small-cell lung carcinoma; (PDPN): Podoplanin; (PTEN): phosphatase and tensin homolog; (PT): phyllodes tumours; (PD-L1): programmed death-ligand 1; (SCC): squamous cell carcinoma; (SPARC): secreted protein acidic and rich in cysteine; (TTF1): thyroid transcription factor 1

No.	Author and Publication year	Tissue	The studied gene
1	Bakheet et al, 2020	Liver	Glypican 3, Glutamine synthetase
2	Bingham *et al,* 2015	Several cancers	PTEN* gene
3	Cioca et al, 2017	HCC	PDPN*
4	Coppock *et al,* 2018	NSCLC*	PD-L1*/CD274 gene
5	Craig et al, 2020	HNSCC (OPSCC)	E6/ E7 transcripts of HPV virus
6	Daneshpajouhnejad et al, 2020	HNSCC*	E6/E7 transcripts of HPV virus
7	Dreyer *et al*, 2013	HNSCC (OPSCC)	E6/E7 transcripts of HPV virus
8	Drumheller *et al*, 2019	HNSCC	E6/E7 transcripts of HPV virus
9	Gafeer *et al*, 2018	NSCLC*	PD-L1 gene
10	Humphries *et al,* 2018	NSCLC	PD-L1 gene
11	Kim *et al*, 2013	gastric carcinoma (GC)	HER2 gene
12	Kim *et al,* 2017	PT *	(SPARC)* gene
13	Kim *et al,* 2018	PT of the breast	B7-H3 and B7-H4 genes
14	Kulkarni et al, 2019	lipoma and liposarcoma	MDM2
15	Mendez-Pena *et al*, 2017	SCC	E6/E7 transcripts of HPV virus
16	Mills *et al*, 2017	Anogenital and H&N neoplasias	E6/E7 HPV transcripts
17	Mirghani *et al,* 2015	HNSCC (OPSCC)	E6/E7* mRNA in HPV* driven cancers
18	Nakajima *et al*, 2017	NSCLC	ALK gene
19	Randen-Brady et al, 2019	HNSCC (OPSCC)	HPV virus
20	Roe *et al*, 2017	Human (Various sites)	EBV and CMV
21	Rooper et al, 2016	HNSCC (OPSCC)	E6/E7 transcripts of HPV virus
22	Rooper et al., 2020	Salivary gland tumours	MYB gene
23	Schache *et al,* 2013	HNSCC (OPSCC)	HPV-16, -18 and -33 E6/E7 transcripts
24	Shi *et al,* 2015	Pulmonary and non-pulmonary carcinomas	Napsin A and TTF-1* genes
25	Tretiakova *et al,* 2018	Metastatic bladder cancer	PD-L1* gene
26	Wang *et al,* 2013	BC*	HER* 2 gene
27	Yu *et al,* 2017	BC	ERα* gene

Table S7 – Extracted data relating to RNAscope characteristics in the included studies

Abbreviations: (DapB): dihydrodipicolinate B.subtilis reductase; (PPIB): peptidylprolyl isomerase B; (Polr2A): RNA polymerase II subunit A; (UBC): Ubiquitin C.

No	label used (chromogenic or fluorescent)	Staining (manual or automated)	Scoring (manual or automated)	Positive control	Negative control
1	Chromogenic	Manual	Manual	Hs-*PPIB*	*DapB*
2	Chromogenic	Manual	Automated	Hs-*UBC*	*DapB*
3	Chromogenic	Manual	Manual	Not mentioned	Not mentioned
4	Chromogenic	Automated	Manual	PPIB/ cyclophilin CYP-S1	Not mentioned
5	Chromogenic	Manual	Manual	Hs-*PPIB*	*DapB*
6	Chromogenic	Manual and automated	Manual	Appropriate controls	Appropriate controls
7	Chromogenic	Manual	Unclear	Not mentioned	Not mentioned
8	Chromogenic	Automated	Manual	Hs-*PPIB*	*DapB*
9	Chromogenic	Automated	Manual	Appropriate controls	Appropriate controls
10	Chromogenic	Automated	Automated (QuPath)	Hs-*PPIB*	*DapB*
11	Chromogenic	Manual	Manual	Not mentioned	Not mentioned
12	Chromogenic	Manual	Manual	Hs-*PPIB*	*DapB*
13	Chromogenic	Automated	Manual	Hs-*PPIB*	*DapB*
14	Chromogenic	Automated	Manual	Not mentioned	Not mentioned
15	Chromogenic	Manual	Manual	Hs-*PPIB*	*DapB*
16	Chromogenic	Automated	Manual	Ubiquitin	*DapB*
17	Chromogenic	Manual	Manual	*UBC*	*DapB*
18	Chromogenic	Manual	Manual	Hs-*PPIB*	Not mentioned
19	Chromogenic	Manual	Manual	Hs-*PPIB*	*DapB*
20	Chromogenic	Automated	Manual	PPIB/ cyclophilin	*DapB*
21	Chromogenic	Manual	Automated	Appropriate controls	Appropriate controls
22	Chromogenic	Automated	Manual	*PPIB*	Not mentioned
23	Chromogenic	Automated	Manual	*UBC*	*DapB*
24	Chromogenic	Manual	Manual	Hs-*PPIB*	*DapB*
25	Chromogenic	Manual	Manual and automated	Not mentioned	Not mentioned
26	Florescent	Automated	Automated	*POLR2A,* and *UBC*	*DapB*
27	Florescent	Manual	Manual	Hs-*PPIB*	*DapB*

Table S8 – Accuracy values of the other techniques

No.	IHC		DNA-ISH		Other Techniques	
	Sensitivity	**Specificity**	**Sensitivity**	**Specificity**	**Sensitivity**	**Specificity**
1	57.00%	56.80%				
5	95%	95%	74%	67%	79%	67%
9	65%	100%				
14	65.00%	82.50%		100%		
15			88%			
16	97%		92%		92%	
19			86%	95.30%	83.50%	89.10%
22	94%	54%				

Supplementary Documents

Supplementary Document 1 - QUADAS-2 Instructions, format, and guidance

A Revised Tool for the Quality Assessment of Diagnostic Accuracy Studies: QUADAS-2

Instructions:

The answer for the Risk of bias could be either; yes, no or unclear
If all of the answers for signalling questions in one domain are "No" or “unclear” or a combination of them, then the risk of bias can be judged "High" For that domain.
The "unclear" answer can be applied when there is not sufficient data for judgment.
The total risk of bias is considered “Low” when three or four out of four domains have a low risk of bias, “Some Concerns” when two of the domains have high or unclear risk of bias, “High” when three or four out of four have high or unclear risks.

QUADAS-2 Format

Study title	
No., Author and year of publication	

Domain 1: Risk of bias due to sample selection	**Comments**	**Response options**
A. Risk of Bias Describe methods of sample selection: 1.1 Was the sample selection consecutive or random?		Y / N / unclear
1.2 The poor quality samples were excluded?		Y / N / unclear
1.3 Did the study avoid inappropriate exclusions?		Y / N / unclear
1.4 Could the selection of samples have introduced bias?		Y / N / unclear No
B. Applicability Did they describe included cases (specimen type, subspecialty, biopsy location)?		Y / N / unclear
Risk-of-bias judgement		Low / High / Some concerns
Domain 2: Risk of bias due to index test		
A. Risk of Bias Describe the index test: 2.1. Was the index test Automated?		Y / N / unclear

If No, how many pathologists analyse the results? More than one?		
2.2. were results interpreted without knowledge of the results of the reference standard?		Y / N / unclear
2.3 If a threshold (classification of the agreement) was used, was it prespecified?		Y / N / unclear
B. Applicability Is there concern that the index tests, its conduct, or interpretation differs from the scientifically followed procedure?		Y / N / unclear
Risk-of-bias judgement		Low / High / Some concerns
Domain 3: Risk of bias due to reference standard		
A. Risk of Bias Describe the reference standard and how it was conducted and interpreted: 3.1 Is the reference standard likely to correctly classify the target conditions (diagnosis)?		Y / N / unclear
3.2 Was the reference standard results interpreted without knowledge of the results of the index test?		Y / N / unclear
B. Applicability Is there concern that the reference standard, its conduct, or interpretation does not match with the review question methods?		Y / N / unclear
Risk-of-bias judgement		Low / High / Some concerns
Domain 4: Risk of bias in measurement of flow and timing		
A. Risk of Bias Describe the time interval and any interventions between index test(s) and reference standard: 4.1 Could the diagnosis flow have introduced bias?		Y / N / unclear
Risk-of-bias judgement		Y / N / unclear
Overall risk of bias judgement		Low / High / Some concerns

Tables A5: QUADAS-2 format

<u>Risk of Bias guidance:</u>
DOMAIN 1: PATIENT SELECTION

A. Risk of Bias

Describe methods of sample selection:

Was a consecutive or random sample enrolled? Yes/No/Unclear

A bias potential can arise when the samples are not selected either consecutively or randomly.

Were the poor quality samples excluded? Yes/No/Unclear

Using samples (tissues) with poor quality might affect the quality of the results.

Did the study avoid inappropriate exclusions? Yes/No/Unclear

Inappropriate exclusions might affect the diagnostic accuracy and give over-optimistic results.

Could the selection of samples have introduced bias? Yes/No/Unclear

The answer of this question should be "No" to get a low risk judgment. As well, it is a subjective question and its answer depends on the reviewer perspective of view.

B. Applicability

Did they describe included cases (specimen type, subspecialty, biopsy location)? Yes/No/Unclear

The description of these details gives a clear idea of what was done and thus reduce the bias risk. The study wouldn't be applicable if they are not mentioned.

DOMAIN 2: INDEX TEST(S)

A. Risk of Bias

Describe the index test and how it was conducted and interpreted:

Was the index test Automated? If No, how many pathologists analyse the results? More than one? Yes/No/Unclear

Automated methods are more reliable, and introduce less bias than the human work. Due to the bias that might be introduced by humans, results evaluation by more than one person will reduce the bias.

Were the index test results interpreted without knowledge of the results of the reference standard? Yes/No/Unclear

The results of the index test should be read blindly without knowledge of the standard reference results. Bias can be appeared if there is a previous knowledge of the results.

If a threshold (classification of the agreement) was used, was it pre-specified? Yes/No/Unclear

The type of the used threshold (scoring scale) should be indicated.

B. Applicability

Is there concern that the index tests, its conduct, or interpretation differs from the scientifically followed procedure? Yes/No/Unclear

If the preparation methods are not conducted regarding the known guidelines, then they might not be applicable to the review

DOMAIN 3: REFERENCE STANDARD

A. Risk of Bias

Describe the reference standard and how it was conducted and interpreted:

Is the reference standard likely to correctly classify the target condition (diagnosis)? Yes/No/Unclear

There is no reference standard for RNAscope as it still a relatively new technique, so the reference standard is the other technique that RNAscope was compared to.

Where the reference standard results interpreted without knowledge of the results of the index test? Yes/No/Unclear

The results of the reference standard should be read blindly without knowledge of the index test results. Bias can be appeared if there is a previous knowledge of the results

B. Applicability

Is there concern that the reference standard, its conduct, or interpretation does not match with the review question methods? Yes/No/Unclear

The reference standard (the gold standard technique) should match with one of the review question techniques to be applicable to the review

DOMAIN 4: FLOW AND TIMING

A. Risk of Bias

Describe the time interval and any interventions between index test(s) and reference standard:

Could the sample flow have introduced bias? RISK: LOW /HIGH/UNCLEAR.

Some subjectivity may be introduced to this question regarding the reviewer point of view.