**Nasal nitric oxide measurement in children for the diagnosis of primary ciliary dyskinesia: Europan Respiratory Society technical standard**

**Short Title:** PCD and nNO: a technical standard

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**“Take home” message**

ERS technical standard for measuring nasal nitric oxide in children with suspected primary ciliary dyskinesia.

158/ 250 word abstract

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**Abstract**

Nasal nitric oxide (nNO) is extremely low in most people with primary ciliary dyskinesia (PCD) and its measurement is an important contributor to making the diagnosis. Existing guidelines and technical standards focus on nNO measurements in older, cooperative children using chemiluminescent analysers. However, measurements of nNO in pre-school children (2-5 years) may facilitate early diagnosis, and electrochemical rather than chemiluminescence analysers are widely used. Pre-schoolers often need different methods to be employed when measuring nNO. Hence a European Respiratory Society Task Force has developed this technical standard as the first step towards standardising sampling, analysis, and reporting of nNO measured as part of the diagnostic testing for PCD in all age groups including preschool-age children. Furthermore, we considered both chemiluminescence and electrochemical analysers that are in use worldwide. There was paucity of quality evidence for electrochemical analysers and sampling methods used in young children, and this manuscript proposes future research priorities to allow updates of this technical standard.

**Background**

Primary ciliary dyskinesia (PCD) is a genetically and clinically heterogeneous syndrome estimated to impact 1 in 7500 people worldwide [1]. Impaired function of motile cilia causes failure of mucociliary clearance leading to symptoms of neonatal respiratory distress of unknown cause at term, daily wet cough from infancy, perennial rhinosinusitis, otitis media with effusion, chronic bronchitis, and bronchiectasis [2, 3]. Approximately 40% of patients have situs inversus totalis, and about 12% have heterotaxy [4]. Male and female subfertility is common [5]. Diagnosis requires access to a combination of specialised investigations which may include transmission electron microscopy, genotyping, high-speed video microscopic analysis of cilia function and immunofluorescence staining of ciliary proteins [6, 7]. Nasal nitric oxide (nNO) concentration measurements contribute to the diagnosis because many people with PCD have reproducibly low levels compared to healthy individuals and to people with other airway diseases [6-9].Although nNO results cannot confirm or refute the diagnosis in isolation, the ERS Diagnostic Guidelines recognises its importance for determining the likelihood of PCD when used in conjunction with other tests or as a screening test [6]. Low nNO levels in PCD were first reported >25-years ago, but the mechanisms underlying low nNO in PCD and its pathophysiological consequences are unknown [8, 10].

The recommended technique for measuring nNO requires the individual to exhale orally against resistance whilst gas is sampled from the nares and nasopharynx [11]. This technique ensures velum closure, thereby avoiding contamination and dilution of nasal gas with air from the lower airways. However, exhaling against resistance (ER) can only be achieved by older, cooperative children (usually >5-years) and alternative sampling methods have evolved to facilitate measurements being obtained from infancy. Simple breath hold (BH) may be possible with some children who cannot follow protocols that include manoeuvres involving ER. Alternatively, measurement of nNO during tidal breathing (TB) is feasible from early infancy [12-15]. Therefore, nNO measurements can technically be performed in all age groups, including infants. However, normal reference values are scarce and diagnostic cut-off values may be difficult to establish in younger children since nNO concentrations are inherently very low during infancy and increase with age during the first years of life [14, 16].

Chemiluminescence analysers are the standard device used to measure nNO levels, relying on the reaction between NO sampled from the patient’s nostril and ozone generated in the instrument. This instantaneous reaction results in emitted electromagnetic radiation in the form of light (photons), which is proportional to the continuously sampled NO molecules [17]. Electrochemical analysers use amperometric sensors to measure the quantity of NO accumulated in a chamber from the current generated by the chemical reaction between the sampled gas and the active sensing material [18].

While American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines recommend that nNO measurements are performed using chemiluminescence analysers [6, 7, 19], a recent worldwide survey reported that electrochemical devices are more frequently used, particularly outside North America [20]. Chemiluminescent analysers produce a real-time display of the NO signal, which is important for quality assurance, and there are standardised, validated methods using these analysers. However, the purchase and maintenance of these non-portable devices is expensive compared to electrochemical analysers. Discriminative nNO values are possible with electrochemical hand-held NO devices [21, 22], but these analysers do not have fully standardised operating procedures, nor have they been tested against established diagnostic criteria in large multicentre trials. No electrochemical analysers have been validated in diagnostic settings, and supporting data was originally derived from the NIOX MINO [21, 22], which is no longer available. While electrochemical analysers are generally more affordable, they do not display real-time NO levels graphically, and hence do not allow selection of optimal nNO measurement plateaus. Thus, whilst electrochemical analysers have benefits in low-resource settings or where portability is important [23], technical improvements as well as studies are still needed to achieve standardised, validated methods for nNO measurements using these devices.

Internationally, clinicians and researchers are using different equipment, breathing manoeuvres, diagnostic cut-off values, and reporting standards. The PCD community has identified the need for standardised and validated methods for nNO measurements as a priority [20].

The aim of this Task Force was to develop a technical standard for the sampling, analysis and reporting of nNO levels as part of the diagnostic testing for PCD in childhood, including in preschool children and infants. We aimed to consider both chemiluminescence and electrochemical analysers that are in use worldwide, and to identify future research priorities.

**Parental perspective**

This verbatim contribution is from a parent representative of the Task Force (MC):

“When our child was having diagnostic tests, we had a little stress with the result of each test. We were correctly explained that the nNO measurement was a very important test because depending to its results, PCD would become very likely or unlikely. It was therefore a lot of stress when we learned that the child's nose must not be obstructed in order to be able to do this test. Our child has chronic rhinitis (as part of her disease) and we did not know how to make it different for this important test.

Another point is that we were very keen to have the results of the diagnostic tests quickly to continue our child’s assessment. I have spoken to parents who have told me that on the occasion of some tests they have been quite harsh / severe (and angry ??) with their child who was not cooperative (because of fear or lack of good will). This can be the case for any test, including nNO test if too much importance is given to it.

In order to decrease the stress related to this specific test, I would suggest to explain to parents that if the result of nNO measurement is not undoubtedly normal (high above the cut-off) it will be necessary to check it on a second occasion, because it is a delicate test and local/nasal disease may alter the results. Also it would be good to specify that 1) deciding to go on with other more invasive tests will also rely on the clinical probability of the disease assessed by a panel of experts 2) the final diagnosis will not rely only on nasal NO but on a range of tests 3) in the meantime, the care of the child would be similarly adapted regardless of the final diagnosis (after the exclusion of differential diagnoses).

If there are technical considerations about the reliability of results depending on the technique used (chemiluminescence or electrochemical), it is very difficult for parents to capture them, and to report them if questioned. Therefore, I suggest that all relevant technical issues be flagged in the nNO measurement report.”

**Methods**

The membership and roles of the Task Force panel and methods are detailed in Supplementary files (Tables E1 and E2 and supplementary text). In brief, to inform the development of the technical standard, we relied on evidence collected through a systematic literature review (January 1994 to December 2021). Each work group reviewed all of the titles, and if relevant the abstracts and then full text to utilise the information relevant to their area of focus. Work groups discussed the manuscripts and then drafted the text which was reviewed by the full task force. Iterative changes were made in a series of virtual meetings until agreement was reached.

**Outcomes from the Task Force**

**Considerations for analysers**

The advantages and disadvantages of chemiluminescence and electrochemical analysers are summarised in Table 1, and although electrochemical analysers can produce reproducible measurements their results are considered as less accurate than reproducible measurements obtained using chemiluminescence analysers (Box 1).

*Sampling rates*

Nasal NO is constantly produced within the nasal cavity. During nNO measurements, air is aspirated from one nostril using an olive attached to the device’s vacuum pump while the other nostril remains open to the atmosphere (Figure 1). The collected air is analysed by the NO gas analyser. The rate at which the air is aspirated can vary depending on the type of device and its settings. Knowledge of this rate is vital for interpretation of the nNO measurements.

Historically, the aspiration (or sampling) rate used to measure nNO has varied widely (from 0.2 to 6.2 L.min-1) [24]. Using the breath-hold technique, Qian et al. demonstrated that the measured NO output is flow-dependent. Slower aspiration flow results in lower NO outputs, possibly because lower, laminar flow does not reach the deeper nasal cavities. A slower aspiration rate takes a longer time for the nNO output to plateau (> 10 sec) [25]. In contrast, turbulent, higher aspiration flow results in significantly higher NO outputs and a shorter time to reach a plateau; high flow rate can cause discomfort (> 1.2 L.min-1) or nostril collapse (> 5.2 L.min-1) [25]. For these reasons, previous ATS/ERS guidelines (2005) recommended limiting the sampling rate to between 0.25 and 3 L.min-1, and documenting the aspiration flow [19]. Since the publication of these recommendations, further evidence for optimal aspiration flow has been sparse. Struben et al. compared three aspiration flow rates of 0.28, 0.7 and 1.2 L.min-1 using the NIOX chemiluminescent analyser and a breath-hold method in adult subjects [26]. They reported that the time to plateau and the resulting mean nNO measurement were significantly different when using these differing aspiration flow rates. These investigators recommended a preferred flow of 0.7 L.min-1 since the NO plateau was reached within 7 seconds and the procedure was well tolerated by adult study participants. In children, using the velum closure method, Beydon et al. reported a significant effect of aspiration flow (0.3 versus 1 L.min-1) on the measured NO output, but could not perform the same comparison using the tidal breathing technique because higher flow disturbed young children [12].

Commercially available devices provide a range of default flows between 0.12 and 0.33 L.min-1 [11, 21, 22, 27]. We suggest that the sampling rate be set at 0.3 or 0.33 Lmin-1. Users should directly measure and record their device’s sampling rate to calculate the output of NO (nL.min-1) (see section “*Interpretation of results- general”*).

*Licensing and regulatory approvals*

Another consideration in device selection may depend on licensing and regulatory approval. Both chemiluminescence and electrochemical devices are licensed for nNO measurements in Europe. Neither chemiluminescent nor electrochemical devices are approved by the US Food and Drug Administration, and the former are used at PCD Foundation-accredited clinical centres, Genetic Disorders of Mucociliary Clearance Consortium sites, or in research settings. Only two chemiluminescence NO analysers suitable for clinical testing (Eco Physics CLD88 device and Zysense NOA 280i) are available for purchase in North America.

**Considerations for consumables**

Measurement of nNO requires single patient use nasal probes with fixed (plastic) or compressible (foam) olives that can be inserted tightly into the nostril to prevent air leakage [28]. Commercially available nasal probes are provided in several sizes, ranging from neonatal to large adult. To prevent contamination of the sampling line with humidity, infectious agents, mucus or other debris, it should be single-use or the nasal probe should have appropriate built-in or in-line filters [29].

For ER manoeuvres, disposable mouth restrictors are required, which can be inexpensive cardboard cylinders with a 1-mm opening at the distal end or a party favour to provide similar resistance [11]. For some commercially available analysers the restriction is achieved by adding a paper restrictor with a 1-mm hole at the back side of the standard bacterial filter that is part of the equipment’s mouthpiece (NIOX VERO). In others, closure of the palate is achieved through exhalation flow control supported by an incentive screen that provides feedback (EcoPhysics CLD88sp). In both cases, as the equipment mouthpiece is used, an additional microbiological filter is required [29].

**Calibration, maintenance, and environmental nitric oxide**

Despite the importance of obtaining accurate and reproducible data, most manuscripts provide minimal information on calibration of devices used in their studies.

Ideally, nNO should be measured in a clinic or hospital space that has low ambient NO levels to avoid potentially artefactual results [30, 31], but a recent survey showed that not all users measure ambient levels [20]. Environmental NO levels below 20 ppb are generally considered acceptable to allow testing to proceed. The 2020 North American Technical Paper on testing suggests that inhalation of NO free air through the nostril open to the air may help if ambient levels are high [27], however, this method has not been validated and may complicate testing of young children. An alternative option is to subtract the ambient NO level from the nNO result [32]. In the absence of any validated method, this Task Force suggests that an estimate of the effect of ambient nNO on the outcome can be made by deducting the ambient nNO from the child’s nNO result. If the ‘corrected’ value is clearly above the diagnostic cut-off value, the result can pragmatically be accepted. If the result is close to (potentially impacting the outcome), or below the cut-off the measurement should be confirmed on another day.

**Example to estimate effect of ambient NO:**

nNO measured with 0.33 L.min-1 sampling flow and an ambient NO of 49 ppb was 281 ppb

nNO output = 281 ppb x 0.33 L.min-1 = 93 nL.min-1 (> cut-off of 77 nL.min-1)

nNO output ‘corrected’ for ambient NO = (281-49)ppb x 0.33 L.min-1 = 232 ppb x 0.33 L.min-1 = 76 nL.min-1 (< cut-off of 77 nL.min-1)

*In this case the result should be evaluated on another day when ambient level is low because the ‘corrected’ output is close to the diagnostic cut-off value.*

In general, users should follow detailed directions for airflow and NO calibrations provided by the manufacturer, although some commercially available electrochemical devices do not provide such information.

When recommended, the flowmeter of the device is calibrated daily, usually using a syringe appropriate to the flow and volume expected during testing. Sites should have an external flowmeter capable of measuring the sampling flow of the NO analyser within a sampling range (0.2 and 0.6 L.min-1) [27].

Regular calibration of chemiluminescent analysers with standardised NO gas with two (high and low) certified calibration gases is recommended by some manufacturers, whilst others require calibration with a high NO standard and with a gas-zero NO (ambient air screened for NO by a module connected to the machine) for low level calibration. The NIOX Flex (Aerocrine AB) (no longer available) required calibration every 14-days, but other chemiluminescent analysers are thought to stay accurate for a longer time and a monthly calibration is appropriate [33], therefore manufacturer’s guidance should be followed. If the nNO values obtained during calibration are unrealistic a leak should be considered, and calibration or measurement should be repeated. Additional calibration should be conducted following any change in the sampling line, inline resistor, or other component of the testing system.

It is not possible to calibrate electrochemical analysers using certified gas, but users can consider biological control testing using a healthy employee with known normal and stable nNO levels.

Maintenance schedules should follow the manufacturer’s guidelines.

**Training of personnel**

While there have been no studies to investigate the impact of operator training, it is evident that operators must understand the performance and limitations of the device used at their centre, as well as the relative accuracy of the result depending on the manoeuvre and the repeatability for a given child (Box 1). One study reported improving success rates measuring nNO during TB in preschool children (6-months - 5-years) with increasing operator experience [13]. As with other physiological measures, operators and technicians must receive formal training in the maintenance and calibration of equipment; to recognise equipment malfunction; select the appropriate manoeuvre for the patient (Box 2); calculate nNO production from acceptable tracings; and interpret the result based on standardised protocols. Training should be conducted by experienced personnel (e.g. BEATPCD, ERN-LUNG PCD core or PCD Foundation), and knowledge should be regularly updated. Training by manufacturers may not be adequate, as in the experience of the authors, the manufacturers may not have a good understanding of the testing requirements.

**Assessment and preparation of the patient before testing**

A clinical history suggestive of PCD [34, 35] must be confirmed before testing, because a low pre-test probability of PCD will lead to an unacceptably high false positive rate [36]. The protocol for measuring nNO is summarised in Box 3.

Information about assessing and preparing patients before nNO measurements is limited. Knowledge gaps exist in the literature regarding the assessment of the nasal passages. Nasal obstruction for any reason (e.g. nasal polyposis) might hamper measurement and result in repeatedly low nNO levels or large inter-nostril differences. Rybnikar et al. reported significantly reduced nNO measurements with increasing size of adenoids in non-allergic non-PCD patients between the ages of 5-18 years; these measurements normalised following adenoidectomy [37]. Therefore, members of the Task Force consider referring children for ENT assessment if an obstruction of any cause is suspected, particularly if the nNO level is low.

Patients should be asked to blow their nose before testing. Gentle saline lavage could be helpful in those unable to adequately clear their nasal passages, taking care not to injure the mucosa. nNO measurements can be performed after a few minutes to allow NO to reaccumulate after the lavage.

Nasal NO should be measured before nasal brushing or biopsy to avoid falsely low readings caused by bleeding, since haemoglobin avidly binds NO and could theoretically reduce measured nNO levels.

Several reports have described reduced nNO measurements in people with primary immunodeficiencies, diffuse panbronchiolitis, and cystic fibrosis [38-42] and although levels are not usually as low as in PCD, these differential diagnoses should be considered (Supplementary table E3). Indeed, at most specialist sites cystic fibrosis must be excluded before performing nNO measures.

There is limited data regarding the effect of infections on nNO. Respiratory tract infections in otherwise normal infants temporarily suppress nNO levels by ≈80% [14]. While children requiring PCD diagnostic testing typically have chronic upper and lower airway symptoms, these data strongly indicate that nNO measurements should not be measured during infective exacerbations. Many centres delay testing for 2-4 weeks following infection [20], but there is no evidence concerning the appropriate duration. In the absence of clear evidence, we suggest testing should be delayed 2-4 weeks after exacerbation symptoms have resolved, and if doubt exists concerning a low nNO measurement the test should be repeated on a separate day.

**Special considerations when measuring in different age groups**

The ERS Task Force agrees with the recently published North American Technical Paper, which focused on individuals > 5years of age [27]. Briefly, that paper concluded that ER is the preferred sampling method, ideally using a mouth resistor or party favour (blow out toy) partially occluded at the distal end. The breath-hold (BH) technique can serve as an alternative in children who cannot perform ER, but it requires subject co-operation to voluntarily close the velum [42-44].

Although ER or any other velum closure method is the preferred method of sampling in school-aged and older children, it is often not feasible in pre-school aged children. Piacentini and co-workers reported that only 14% of healthy children between the ages of 2-5 years could actively cooperate [45]. The TB method is the most feasible method for infants and younger children. If velum closure is achieved, BH and ER show similar repeatability in children whilst tidal breathing, a non-velum closure method, shows greater variability [46].

Gupta et al. reported successful TB measurements in 69% of a non-PCD cohort of children between the ages of 6 months and 5-years [13]. Marthin et al. found that nNO was reliably measured during TB in 95.2% of children < 6-years of age, which highly exceeded the success of prolonged breath hold velum closure for 30-40 seconds or short intervals of velum closure obtained by blowing repeatedly in a party favour [42]. Similarly, Beydon et al. found that ability to perform velum closure nNO manoeuvres increased with age, with a 20% success rate in those under 3-years of age, increasing to 83% in 3 to 6-year-olds, and 98% in children > 6-years of age [12].

Feasibility and success rates for infant nNO measurements using the TB method have been described in two studies [14, 15]. Buechel et al. successfully obtained nNO measurements from 100% of 62 neonates during natural sleep using chemiluminescence assays, whereas the success rate was lower (85.5%) when they used an electrochemical device [15]. A similar high success rate (99.6%) was reported by Marthin et al. in 44 infants 2-weeks to 2-years old using a chemiluminescence analyser [14].

All studies reported extremely low nNO concentrations in healthy infants [14-16, 47, 48], with levels increasing rapidly during the first 18-months [14, 16, 48], then more gradually until 12-years of age when they reach adult levels [30]. Since healthy infants and young children can have low nNO levels, reference and cut-off values are needed at different ages to discriminate children with PCD from those without. Normative data remain limited, largely based on small, single centre studies usually involving healthy infants [14, 16, 48] and neonates [15].

Because of extremely low levels of nNO in healthy infants <12-months and the paucity of normative data, Task Force members do not measure nNO diagnostically, but some use it as a research tool in this age group until age-related testing standards and diagnostic cut-off values are established. For children older than 12-months, an experienced technician should assess the likelihood of a child’s ability to achieve a manoeuvre based on age and cognition. Again, assessment of results in children under 5-years of age is limited by the lack of normative and cut-off values for disease and should be interpreted with caution (Supplementary Tables E3 and E4), referring to a PCD centre and repeating the measure when the child is older is recommended if doubt exists.

**Standard operating procedure (SOP) for performing a measurement using different respiratory manoeuvres**

The order in which different nNO test manoeuvres are undertaken to determine feasibility for a specific child does not impact the result and the technician should decide which manoeuvre to use based on the likelihood of success (Figure 2). The preparation and measurement of nNO are summarised in Box 3.

***General considerations for measuring nNO in all age groups***

For the measurement:

* Children should be comfortably seated on a chair or a parent’s lap. Some infants will be calmer staying in their pushchairs, or they might sleep during the procedure (Figure 1).
* The technician should explain the procedure to the patient. Demonstrating the procedure and practising (e.g., positioning of the olive, breathing manoeuvre) before the actual testing may avoid repetitive testing (Figure 3).
* A nasal olive appropriate for the child’s size should be inserted into one nostril to form an airtight seal, avoiding sampling of the room’s air. The olive is maintained in place by the patient whenever possible, or by the technician or a parent in the case of young children. To avoid leaks when testing young children, it might be beneficial to support the child’s head by placing a hand behind the head or to sit the child on the parent’s lap, with their back resting on the parent’s torso. The other nostril should be unobstructed (Figure 1).

Further measurements should be undertaken from the same nostril until two nNO levels within 10% (20% for TB measurements) of each other are obtained, with 30 seconds of rest between measurements [11, 27, 46]. The same procedure is repeated using the opposite nostril aiming for 10% inter-nostril repeatability (30% for TB measurements) [11, 27, 46]. Single measurements or assessing a single nostril can be prone to error [46].

It may be impossible to attain ‘ideal measurements’ in children, e.g., the child may be too young to achieve ER, only a single measure may be attained in one nostril, and chemiluminescence may not be available. The Task Force has therefore developed a scoring system to denote acceptability where Grade A is the ideal nNO test, and Grade F is the least reliable (Box 1; and explained in supplementary files).

*Expiration against resistance manoeuvre*

The methods for measuring nNO using an ER manoeuvre have been described in a recent technical standard for older children and adults [27]. In brief, testing is ideally performed using an analyser that allows the technician to view the nNO concentration curve in real time and manually determine the plateau value following the measurement, but this is only possible using a chemiluminescence analyser [27, 49].

The technician should first measure and record the sampling flow as explained above. After checking the equipment and assessing the ambient NO levels, the technician attaches the flow sampling line to the nasal olive probe and filter and inserts the probe into the patient’s nostril. A resistor mouthpiece is then held in their mouth with a targeted expiratory mouth pressure of at least 10 cm H2O in adults and 5 cm H2O in children [19]. Alternatively, a party favour (blowing toy taped closed at the distal end to prevent too rapid an exhalation) can be used to create resistance (Figure 1c). The individual should then inhale to near-total lung capacity before beginning a prolonged exhalation manoeuvre, sealing the resistor tight with their lips, blowing in a steady, low-flow manner until they are directed to stop by the technician (i.e. when the curve shows an acceptable plateau of at least 3 seconds).

The technician should manually choose the plateau rather than using the automated software [27]. Acceptable nNO plateaus are 3-seconds or longer in duration and there should be less than 10% variation (between the minimum and maximum), using the maximum attainable mean plateau values [27] (Figure 4). A low nNO level should ideally be confirmed by testing during TB (Figure 4).

If an electrochemical device is used, the manoeuvre is similar to a chemiluminescent device, except that reliable feedback showing the real-time NO curve is not possible. With the electrochemical device, the child exhales for the duration set by the machine, which should not be less than 10-seconds [49]. The plateau duration for electrochemical devices displaying nNO tracings (*e.g*., NIOX VERO) have not yet been established. Moreover, electrochemical analysers without a display of a NO tracing do not allow manual selection of a technically acceptable plateau, but provide the mean NO measured from part of or from the entire sample. For instance, the manufacturers stated that for the NIOX MINO set at 5 mL.s-1, the last 30-seconds over the 45-second sampling time was measured.

Typically, the measured nNO values obtained from the two nostrils should be similar. Greater variation may indicate nasal obstruction, e.g., polyps [12]. Alternatively, the sampling line or an in-line resistor may have become obstructed. When variation occurs, the technician should remeasure the sampling flow following testing to determine whether the value has increased compared to the pre-test value. The test should be repeated after secretions are cleared or the element replaced [27].

*Breath-hold manoeuvre*

This manoeuvre is an alternative to the preferred method of exhalation against resistance and may be used for patients who cannot perform ER. Details are similar to ER method, except for the breathing manoeuvre itself.

The patient is instructed to take a deep breath to total lung capacity. They then ideally perform velum closure, in which they close the glottis and perform a Valsalva manoeuvre to elevate the hypopharynx and close the soft palate. If measured, nasal CO2 from the opposite (free) nostril can confirm velum closure has been achieved (CO2 = 0 %) [19]. Failure to achieve velum closure can falsely reduce nNO measurements due to contamination with exhaled air from the lungs and oropharynx, diluting NO in the nasal sample.

When using a chemiluminescent analyser, the BH manoeuvre should be maintained to achieve a plateau of > 3 seconds with < 10% variation between minimum and maximum values of the plateau. In contrast, electrochemical analysers have a predetermined duration for the sampling, and patients need to hold their breath until sampling is completed, which may be difficult for some. Using older electrochemical devices (*e.g*. NIOX MINO), the sampling duration depended on flow (90-sec at 2 mL.s-1 and 45-sec at 5 mL.s-1) [21], while in the newer versions (*e.*g. NIOX VERO) the sampling time is 30-seconds at 5 mL.s-1.

*Tidal breathing method*

Nasal NO measured during tidal breathing has been described in more than 15 study reports or reviews [9, 12-16, 22, 32, 42-44, 48, 50-52] where the method has been used among infants, children, and adults, whether healthy or patients with suspected PCD, definite PCD or other respiratory conditions (Supplementary files Tables E3 and E4).

Measurements have been made predominantly in an upright sitting position or, in the case of infants, lying down and possibly during sleep [14-16, 42, 50] (Figure 1). During tidal breathing, which can be performed with open or closed mouth, the sampling described in the literature varies widely from a few seconds to a full minute [9, 12-16, 22, 32, 42-44, 48, 50-53].

TB nNO results have most often been reported as the mean of either three [14, 22, 42, 43, 51, 53] or five peaks from the tracing curve that displays regular breathing [12, 16, 50] (Supplementary Table E3). Peaks were chosen based on the highest values, sometimes needing to be consecutive (reflecting the regular respiration) or sometimes based on reproducibility criterion. In other studies, the result was the average over the time of the sampling [12, 13, 44, 45, 48].

To standardise the method for measuring TB nNO using a chemiluminescence analyser, we recommend that the mean of 3 to 5 maximum observed peaks is reported during a period of regular breathing over 30 seconds. If the child is uncomfortable and breathes irregularly, then a break before a new trial is preferable than the continuation of the measurement. If, for no clear reason (calm child breathing regularly) successive peaks are still not regular in height before 30 s the recording can be extended after 30 s. The peaks should be within 20% or 10 ppb whichever is the greatest [46]. (Figure 5) It is a limitation of electrochemical devices that you can only report the average nNO sampled during the time set by the machine which is always lower than the peak values and requires specific cut-off values or algorithms [12].

*Other manoeuvres such as humming, slow nasal or oral expiration without velum closed*

Other techniques, such as humming, and slow nasal or oral expiration without velum closure have been tested in smaller, single site studies [43, 44, 54]. These approaches require subject cooperation and reproducibility, which may be difficult for some patients [42]. The reproducibility and accuracy of these measures have not been determined, and large-scale validation studies would be needed.

*Causes of flawed nNO results according to the methods of measurement*

Considerations about flawed results and troubleshooting are provided in the Online Supplemental Material text and Supplementary Figures E2-5.

**Reporting and interpretation of results**

The minimum information to include in a report is summarised in Box 4, and in a sample proforma for data collection. (Supplementary Figure E6). Interpretation of the result is summarised in Box 5.

*Reporting and interpretation of results - general*

Normative data are generally lacking, particularly for young children, electrochemical devices, and TB manoeuvres (Supplementary files Tables E3 and E4).

Evidence is based on studies using chemiluminescence analysers and further research is needed to confirm whether electrochemical devices are equivalent.

The difference between ER or BH nNO values obtained using one or the other analyser should be small and only due to the difference in the chemical process calculating NO molecules. However, the difference between TB nNO measured using chemiluminescence or electrochemical devices is additionally altered by the difference in the part of the sampling used to calculate the result (mean of peaks versus mean of a period of TB).

The highest of two repeatable nNO measurements for each nostril should be recorded. In around 75% of cases, nNO variability in the same nostril is expected to be < 10% for measurements performed with the velum closed and < 20% for measurements performed during tidal breathing [46](Figure 4). The nNO levels in parts per billion (ppb) obtained from each nostril should then be reported along with inter-nostril repeatability, since the result reliability will decrease with increasing variability, especially with electrochemical analysers where no NO curve is displayed (Box 1).

Having determined the final nNO result in parts per billion for each nostril, the highest nNO value from the two nostrils is converted to nanolitres per minute to standardise for the sampling rate using the equation:

***Standardised nNO value (nL.min-1) = nNO concentration (ppb) x flow sampling rate of analyser (L.min-1)***

For example, using a CLD88sp analyser (EcoPhysics) with a sampling rate of 0.33 L.min-1, if the final averaged nNO concentration is 500 ppb, the final standardised value is 500 x 0.33 = 165 nL.min-1. The sampling rate should be recorded as a subscript of nNO to allow between-result comparisons, e.g., TB nNO0.33.= 500 ppb; or TB nNO0.33= 165 nL.min-1.

The standardised nNO from the highest result from the nostril with highest reading, should then be compared to reference data or cut-off as described below, taking into account age, breathing manoeuvre, ambient NO and analyser.

When interpreting the results, it is important to remember that although nNO is an excellent test during the PCD work-up, false positive and false negative results occur. There is as an increasing number of PCD genes associated with nNO levels above the 77nL.min-1 cut-off, which will need to be revisited in multinational studies [55, 56]. We recommend that all low or doubtful results are confirmed on a different day.

*Interpreting results - exhalation against resistance*

Several single centre studies have evaluated reference data and nNO values obtained using ER in diagnostic settings (Supplementary files Tables E3 and E4). In a multicentre study, Leigh et al. reported that for children 5-years and older, nNO measured using chemiluminescence is typically > 250-300 nL.min-1 in healthy controls and < 77 nL.min-1 in PCD [11]. This study reported that with a cut-off of 77 nL.min-1, the sensitivity was >98% and specificity was >99.9% across all age groups to identify PCD. Other studies, primarily using similar cut-off values, reported variable but generally good accuracy.[9, 22, 35, 40, 42, 43, 51, 57-60] (Supplementary files Table E4). There is a strong agreement between repeated ER measurements made 1 to 4 months apart (ICC 0.80; 95% CI 0.61 to 0.89) [43].

To be consistent with the North American Technical standard, for nNO measurements performed during exhalation against resistance with a sampling rate of 0.3 or 0.33 L.min-1, we currently suggest using a cut-off of 77 nL.min-1 as part of the PCD diagnostic workup. This assumes that most cooperative children will be 5-years or older.

*Interpreting results - breath hold*

Several studies have evaluated nNO measurements obtained through the BH method in PCD (Supplementary files Tables E3 and E4). Mean BH nNO values may be lower than ER nNO measurements, with larger standard deviations if the velum is not completely closed resulting in dilution from the lower airway. Despite this, its discriminatory value is comparable to ER, and BH provides an adequate alternative to ER using the same cut-off values. BH nNO demonstrated very strong agreement between measurements repeated 1 to 4 months apart (ICC 0.85; 95%CI 0.70 to 0.92) [43].

We recommend a cut-off of 77nL.min-1 for nNO measurements during breath hold (sampling rate 0.3 or 0.33 L.min-1, and assuming around 5-years or older). If the level is lower than 77nL.min-1, we recommend that a tidal breathing manoeuvre additionally be performed to exclude a false low reading (e.g., if velum closure was not attained).

*Interpreting results - tidal breathing*

Several studies have shown that nNO values are lower during TB than during ER or BH manoeuvres [9, 12, 21, 22, 42, 43, 45, 48] and specific cut-off values are needed to discriminate PCD from non-PCD patients when using this technique in the diagnostic setting (Supplementary Table E4). Furthermore, values are slightly lower during TB with the mouth closed than when the mouth is open [43]. A study of children (median age 7-years; IQR 4.7-11.0) undergoing PCD diagnostic testing reported that nNO levels measured during TB were two-thirds of the ER values with excellent correlation between methods, as was also found by Boon et al. [50, 51]. In the former study, a cut-off value of 44 nL.min-1 (sampling rate of 0.3 L.min-1) was calculated to identify patients with PCD [50]. In children under 5-years of age (n=90), TB sensitivity and specificity were 76.9% (95% CI 54; 99.8) and 85.7% (95% CI 77.9; 93.5) respectively [50]. The low sensitivity was explained by nNO levels above the cut-off value in three children (mutations in *RSPH1*, *CCDC103* and *FOXJ1*). Boon et al. also reported lower specificity when measuring nNO during TB compared to ER [51]. The variability of nNO measured during TB is larger than in manoeuvres ensuring velum closure for PCD and controls, reducing the discriminatory ability of the test [9].

As previously discussed, infant nNO levels are extremely low at birth, and increase throughout the first few years of life, most rapidly during the first 6 months [14, 16]. In a cross-sectional study of 42 healthy infants less than 1-year of age, Adams et al. reported low mean nNO levels (< 15 nL.min-1) in neonates, that increased to approximately 60 nL.min-1 by 12-months of age [16]. Similarly, Marthin et al. reported median values of 15 nL.min-1 at 2 weeks; 42.6 nL.min-1 at 8 months; 58.7 nL.min-1 at 18 months; and 93.4 nL.min-1 at 24 months [14] in a longitudinal study of 44 healthy infants recruited at birth. As expected, limited data suggest that nNO levels are even lower in the first few months in infants with PCD and remain very low [14-16].

In older children and adolescents (ages 5-18 years), Mateos-Corral et al. found very strong agreement of TB measurements when repeated after 1 to 4 months (ICC 0.88; 95%CI 0.76 to 0.94) [43]. Gupta et al. showed similar reproducibility over 24 hours in 21 children under 5-years of age (ICC 0.88; 95%CI 0.71 to 0.95) [13].

Small, single centre studies measuring the mean of 3 to 5 peaks with a sampling rate close to 0.3 L.min-1 suggest a cut-off of 30 nL.min-1 (ie. lower limit of normal) when measuring nNO during TB in children between the ages of 1-2 years, and for children >2-years, we suggest 44 nL.min-1 cut-off (ie. best cut-off established in children 4 years and older) [14, 16, 50]. For the mean of 30 s of TB in children 4 years and older, the cut off would be slightly lower (40 nL.min-1) [12,22]. However, it is important to remember that published, multicentre studies validating these cut-off values are lacking. Repeated measures over time are needed to confirm low results in young children.

**Gaps in knowledge and future directions**

Despite longstanding use of nNO measurements in the diagnosis of PCD, many issues remain unresolved regarding these measurements and research is urgently required. In particular, although electrochemical analysers are the most commonly used devices in Europe, no research has been conducted in the diagnostic setting. The following research is urgently needed:

* Reference data is required, particularly
  + in young children and infants
  + using electrochemical devices
  + during tidal breathing manoeuvres.
* The accuracy of nNO measurements in diagnostic settings and the optimal cut-off values taking into account
  + The ages of patients
  + The genetic cause of PCD
  + The analyser type (chemiluminescence or electrochemical)
  + The breathing manoeuvre (ER, BH, TB)
* The effect of ambient nNO on readings, and how best to manage high levels of ambient nNO levels when reporting and interpreting results.
* How long to delay measuring nNO after a respiratory tract infection? Current practice is 2-4 weeks.
* How long to wait between with-in occasion repeated measurements?
* The extent that preschool children with frequent adenoidal hypertrophy may have false low nNO values and how to “correct” for this in routine practice.
* Other necessary studies include: maintenance frequency, calibration frequency, the influence of sampling tube diameter on sampling rate, the long-term stability of NO-sensors, and whether biological control testing can be used for electrochemical sensors where standardised calibration is not possible.
* It is also unclear if nNO measurement should be repeated to confirm results and, if so, what is the best timing of this repeat measurement (e.g. 1 month later).

**Conclusions**

Nasal nitric oxide is a relatively quick and inexpensive test which contributes to the diagnosis of PCD. Previous technical guidelines have focussed on measurements using a chemiluminescence analyser during velum closure manoeuvres. A recent global survey demonstrated that many centres instead use electrochemical analysers, which are less expensive to purchase and maintain [20]. Also, non-velum closure manoeuvres are commonly used, particularly with young children. Despite widespread use, there are many gaps in our current knowledge regarding use of electrochemical analysers, and the role of nNO measurements in the diagnostic woork-up of PCD in pre-school children. A Task Force of experts therefore developed this technical standard, relying more on experience rather than extensive multicentre evidence. The Task Force highlighted where research is urgently needed to facilitate future evidence-based standards.

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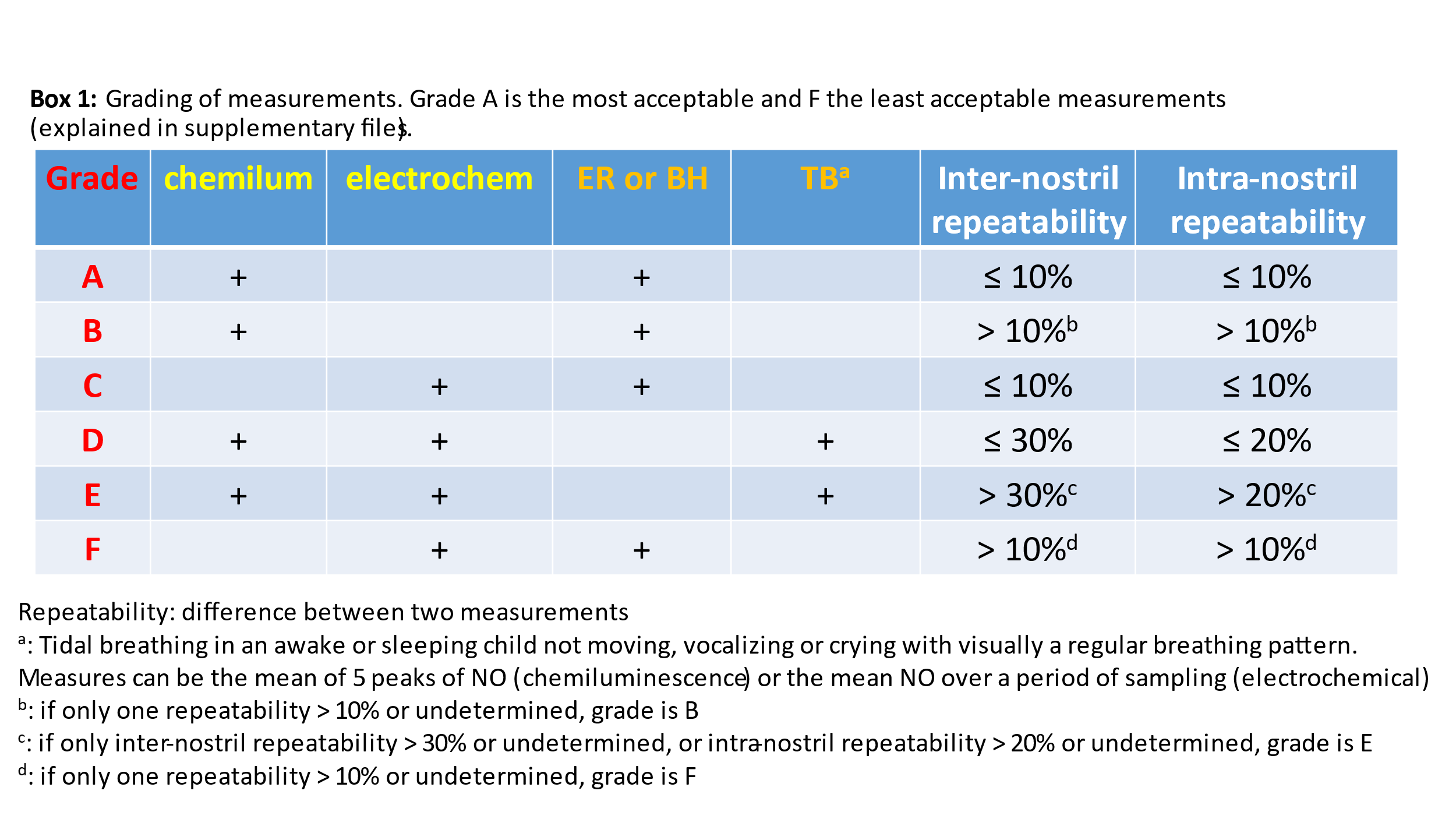
**Table 1:** The advantages and disadvantages of chemiluminescence and electrochemical analysers

|  |  |  |
| --- | --- | --- |
|  | **Advantages** | **Disadvantages** |
| **Chemiluminescent Analyser**  e.g.  CLD88sp (EcoPhysics/ EcoMedics, Switzerland)  Sievers/Zysense NOA-280i (General Electric Analytical Instruments, USA)),  NIOX Flex (Aerocrine AB, Sweden),  LR2000 (Logan Research Ltd, UK) EVA 4000 (SERES, France) | Higher accuracy (eg. < 1 ppb with 1% linearity from 0.1 to 5000 ppb, CLD 88® Eco Medics) | Expensive to purchase and maintain (high cost per test for centres performing a limited number of measurements) |
| Continuous measurement and real-time display of NO test sample | Need for regular calibration and preventative maintenance |
| Allows for identification of the measurement endpoint – stable plateau (ER, BH) or regular peaks (TB) without a fixed sample collection minimum time requirement | Requirement for increased operator training and expertise |
| Online, real-time display allows identification and recording of unreliable measurements (nares leak, contamination from lower airways), aiding the validation of the result | Difficult to transport, and although an “offline method” of measurement allows for remote sampling, it loses the advantage of the real-time trace |
| Ambient NO can be measured and recorded prior to each test occasion |  |
| Rigorous testing in young people 5-years and older with published, validated diagnostic or screening cut-off values |  |
| **Electrochemical Analyser**  e.g., Circassia (formerly Aerocrine AB) NIOX MINO (discontinued 2018) and NIOX VERO models | Simple to use and requires no calibration | Output parameter of mean nNO lacks controlled, multicentre studies validating reference ranges for interpretation (mean values acquired during TB are significantly lower than the peaks values) diagnostic or screening cut-off value. |
| Cost effective solution for low volume sites | Requires uninterrupted sampling for a fixed time that can be problematic in young children due to interrupted flow (sniffing, crying) or difficulty maintaining the desired technique for the fixed duration of the test. |
| Smaller, portable device allowing nNO measurement at different sites | Difficult to detect unreliable measurements (leak, lower airway contamination) without a real-time sample display |
|  | NIOX VERO relies on a cost per test model with the analyser requiring replacement after a set number of tests, or a set timeframe. Discourages recording of ambient NO levels and repeated measurements. |
|  | Lower accuracy e.g., ± 5 ppb for values < 50 ppb and 10% for values > 50 ppb, (Niox Vero®, Circassia\*) |
|  | Inconsistent training and standard operating procedures provided by manufacturers |

ER: Expiration against resistance; BH: Breath-hold; TB: Tidal breathing; ppb: parts per billion

\* <https://www.niox.com/en-us/pdf/000249-11%20NIOX%20VERO%20Labeling%20summary.pdf>

**Summary boxes**



**Box 2: Considerations for nNO in different age groups**

* **<12-months,** extremely low in healthy infants, therefore research tool only, not diagnostic
* **<5-years**, interpret with caution and refer to PCD centre if in doubt:
  + Levels in healthy children < 5 years are lower than older healthy children
  + Limited normative data
* To choose manoeuvre:
  + ER if compliant (usually > 5-year)
  + Breath hold if compliant but unable to achieve ER
  + TB if non-compliant or unable to achieve ER/BH

**Box 3**: **Measuring nasal nitric oxide**

**General**

1. Check equipment and calibrate if needed.
2. Assess the child to determine likely attainable breathing method and olive size.
3. Explain the procedure and practice.
4. Attach flow sampling line to olive with attached filter.
5. Position olive in nostril with no leak; other nostril open.
6. Obtain x2 nNO levels from first nostril (ER/BH: ideally within 10%; TB 20%)
7. Repeat in second nostril (ER/BH ideally within 10%; TB 30%)

**Exhaled against resistance**

* Resistor mouthpiece 5-10 cm H2O; or party favour (blow out toy taped closed at the distal end)
* Ideally, with chemiluminescence device:
  + Slow oral exhalation against resistor until a plateau > 3 seconds with < 10% variation (min/max)
  + View real-time and manually determine optimal plateau
* Alternatively with electrochemical device:
  + Slow oral exhalation for duration set by machine (must be > 10 seconds)
  + If available, the NO tracing is not real-time but can be used to manually select the plateau (> 3 seconds with < 10% variability). Alternatively accept the machine’s result.

**Breath hold**

As for ER except:

* Instruct child to inhale to total lung capacity
* Achieve velum closure whilst holding breath by closing glottis and performing Valsalva manoeuvre
* If measured, CO2at the open/free nostrilshould be zero

**Tidal Breathing**

* During > 30 seconds of stable tidal breathing:
  + Ideally, with chemiluminescence device, manually choose the mean of 3 to 5 peaks (can be non-consecutive and should be <20% variability or 10 ppb, whichever is greater).
  + With electrochemical, if possible, select the peaks from a tracing. Alternatively report the result calculated by the analyser (this is the average nNO sampled, not the mean of peak values and is therefore lower than the chemiluminescence result).

**Box 4: Reporting**

As a minimum the following should be included in a report (sample proforma Supplementary figure E6)

* analyser model
* sampling rate
* ambient NO
* testing method used
* x2 repeatable nNO results from right nostril
* x2 repeatable nNO results from left
* Intra-nasal repeatability (ideally ER/BH <10% variation; TB<20%)
* Inter-nostril variability (Ideally ER/BH ≤ 10%; TB ≤ 30%)
* Any technical or other noteworthy comments
* **Final result in ppb=** highest result from highest nostril (**MINUS** ambient level if >20 ppb)
* **Final standardised value (nL.min-1) =** final result in ppb **x** sampling rate of analyser (L.min-1)

**Box 5: Interpretation of results**

**General**

• If ambient NO> 20 ppb, estimate its effect on the result by subtracting the ambient from patient’s NO as described in the text. If the final result is well above the cut-off, it can be accepted. If it is close to the cut-off (based on local experience of variability) or an accurate result is needed it should be repeated another day.

• False positive and false negative results can occur

**Exhaled against resistance and breath hold (chemiluminescence or electrochemical devices)**

• Cut off is 77nL.min-1 with sampling rate close to 0.3 L.min-1

• If < 77nL.min-1 ideally perform tidal breathing to exclude false positive result

• If < 77nL.min-1, further PCD diagnostic testing is indicated (consider repeating nNO first)

**Tidal breathing**

Reference data is limited. In the experience of Task Force experts

o 1-2 years cut off 30 nL.min-1

o > 2-years cut off 44 nL.min-1 for mean of peaks (chemiluminescence) or 40 nL.min-1 for mean of 30 s of tidal breathing (all types of device)

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