Exhaled Nitric Oxide as a Diagnostic Test for Asthma: Online Versus Offline Techniques and Effect of Flow Rate

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Online versus Offline Techniques and Effect of Flow Rate

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Measurement of the fraction of exhaled nitric oxide (F_ENO) has been proposed as a noninvasive assessment of asthmatic airway inflammation. The influence of the expiratory flow rate during the collection maneuver on the ability of F_ENO to discriminate healthy subjects from those with asthma is unknown. We compared online and offline measurement of F_ENO at different flow rates. F_ENO was collected with expiratory flows of 50–500 ml/second in 34 patients with asthma (PC_{20} of less than 8 mg/ml) and 28 healthy subjects (PC_{20} of more than 10 mg/ml) using offline collection techniques. In a subgroup of 18 individuals with asthma and 17 healthy subjects, we additionally measured F_ENO at multiple expiratory flow rates (47–250 ml/second) using online methods. F_ENO fell with an increasing expiratory flow rate; F_ENO was higher in subjects with asthma as compared with healthy subjects at each flow rate studied with both techniques ($p < 0.001$). Receiver operating characteristic (ROC) curves for the diagnosis of asthma indicated that F_ENO is a robust discriminator between individuals with asthma and healthy subjects (area under the ROC curves 0.79 ± 0.06 to 0.86 ± 0.06, p for significant discrimination < 0.0001). Neither expiratory flow rate nor collection technique (online versus offline) significantly altered this discriminatory capacity (area under the ROC curves = 0.84 ± 0.07 with the slowest online method versus 0.80 ± 0.07 with the fastest offline method, p = 0.46). These data indicate that the choice of expiratory flow rate and collection method can be based on practicality and patient comfort without compromising the utility of this test for asthma.

Keywords: nitric oxide; asthma; diagnosis

In comparison to normal individuals, the fraction of exhaled nitric oxide (F_ENO) is elevated in subjects with asthma, and these elevated levels have been shown to vary with disease activity and in response to antiinflammatory therapy (1–5). These observations have prompted several authors to suggest that F_ENO may be a noninvasive marker of asthmatic airway inflammation (1, 6–8). In this regard, Chatkin and colleagues have previously demonstrated in a population of subjects with chronic cough that F_ENO discriminated well between those with and those without asthma (9).

Many technical factors influence measured F_ENO values, including method of collection (offline reservoir bag or online), time of day, and expiratory flow rate. Among these, expiratory flow rate had been demonstrated to have the most dramatic effect, with F_ENO declining with an increasing expiratory flow rate. Furthermore, collection techniques that use slow expiratory flow rates produce NO higher values and larger part per billion differences between normal and individuals with asthma (10–13). It has therefore been proposed that these low-flow methods will allow for greater discrimination between health and disease (12, 14). In addition, as standard offline measurements comprise the entire expirate (including dead space gas), do not allow for the real-time monitoring of the exhalation and exclusion of technically poor maneuvers, and are potentially subject to contamination through leaks in the collection bag, they have been considered a second choice to online measurements (15). However, slow online collection maneuvers may be uncomfortable for some subjects (16), and offline collection techniques allow for remote collection (i.e., in several clinics) with centralized use of a single analyzer greatly expanding the potential use of this new measurement. We conducted a systematic investigation of the effects of collection technique (online versus offline) and flow rates on the ability of exhaled NO to discriminate between individuals with and without asthma. In this study, we quantified and compared the ability of exhaled NO measured offline at several expiratory flow rates to distinguish individuals with asthma from healthy subjects and compared it with online measurements made at similar flow rates.

METHODS

Subjects

Adult nonsmokers with and without asthma were enrolled. Subjects with asthma had a history of asthma (17), with either a 12% improvement in FEV_1 after inhalation of a β agonist or a methacholine PC_{20} of 8 mg/ml or less. These individuals were using no asthma medications except for short-acting bronchodilators, which were withheld for at least 8 hours before all testing. No systemic or inhaled corticosteroids had been used within 8 weeks of enrollment. Healthy (without asthma) subjects had no history of asthma, normal spirometry, and a methacholine PC_{20} more than 8 mg/ml. All subjects had been free of upper respiratory infection for at least 6 weeks. The study protocol was approved by the Human Research Committee at the Brigham and Women's Hospital. All subjects gave their written informed consent.

Protocol

The study comprised a single visit during which subjects provided exhaled gas for NO determinations at several expiratory flow rates using offline techniques as described later here. A subgroup, approximately half of the full study population, additionally provided exhaled gas for online measurements as described later here. In all cases, the order of expiratory flow rates used and the order of measurement technique (online versus offline) were randomly determined to avoid an ordinal effect. As spirometry has been associated with alterations in F_ENO, no FVC maneuvers were performed within 24 hours of the study visit (18–20).

Offline NO Collection and Analysis

The technical aspects of the offline collection have been described previously and are consistent with those specified in the recommendations of the American Thoracic Society (21, 22). Target flows at 10 mm Hg pressure through the system were verified by a flow and pressure calibration analyzer (model RT-200; Timeter Instruments, Lancaster, PA). Collections were made at 50, 100, 200, 350, and 500 ml/second in triplicate. The NO concentration in the Mylar bag was measured using a chemiluminescence analyzer (model 280; Sievers, Boulder, CO) within 12 hours of collection. The median value at each flow rate was reported.

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TABLE 1. BASELINE CHARACTERISTICS OF THE STUDY SUBJECTS

	Asthmatics (*n* = 34)	Normals (*n* = 28)
Age, years ± SEM	29.6 ± 1.6	27.3 ± 1.3
Sex, % female	59	57
FEV_1, l, ± SEM	3.16 ± 0.18	3.64 ± 0.1
FEV_1, % predicted, ± SEM	85 ± 3.1	97 ± 1.7
PC_{20}, mg/ml, geometric mean, IQR	1.03 (0.22, 4.73), (n = 26)	N/A*
FEV_1 BD response, % improvement ± SEM	28.6 ± 4.3 (n = 8)	

Definition of abbreviations: PC_{20} = Provocative concentration of histamine aerosol causing a 20% decrease in FEV_1.
* By definition, all normal subjects had PC_{20} of more than 8 mg/ml (100% > 10 mg/ml).

Online NO Collection and Analysis

Online measurements were performed according to ATS recommendations with the exception of the variation in expiratory flow, as described later here, using a NOA 280 (Sievers) (22). While standing, subjects inhaled to TLC from a source of NO-free air and then exhaled into the analyzer mouthpiece attached to a one-way valve. Expiratory resistance was provided by needles of varying caliber attached to the expiratory limb of the apparatus (Sievers restricted breath kit; Sievers). At a pressure of 10 mm Hg, the flow through the system was 43, 108, 210, and 250 ml/second.

Statistics

Differences in data obtained from the same subjects were analyzed by two-tailed paired *t* tests (for normally distributed data) or by the Wilcoxon signed-ranks test (for nonnormally distributed data). Differences between subjects with asthma and healthy subjects were analyzed by two-tailed *t* tests (for normally distributed data) or by the Mann-Whitney U test (for nonnormally distributed data). Areas under the receiver operating characteristic (ROC) curves were compared using the Hanley and McNeil method (23). Construction of the ROC curves and all statistical tests were performed using Analyze-it, version 1.61 (Analyze-it, Inc., Leeds, UK).

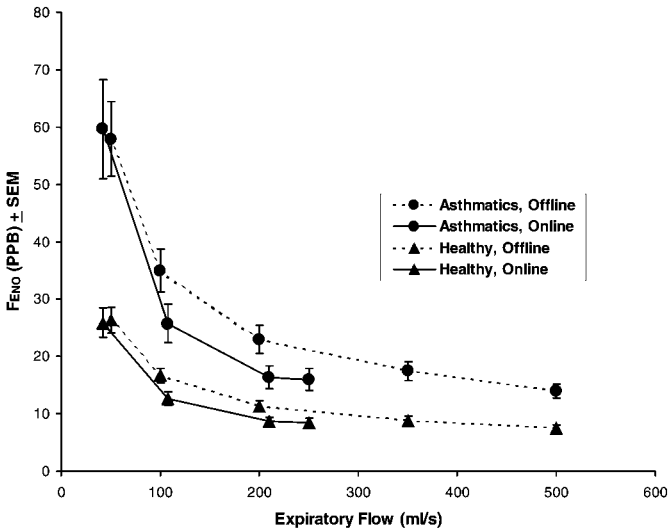

Figure 1. Effect of flow rate and collection techniques on measured FENO in healthy subjects (n = 34) and subjects with asthma (n = 28). In all subjects, FENO fell with an increasing flow rate. FENO was lower at each measured flow in comparison to the next lowest rate ($p < 0.001$ for all comparisons with the exception of online 210 versus 250 ml/s, $p = 0.58$ and 0.27 for healthy subjects and subjects with asthma, respectively). At each rate and with both collection techniques, FENO was higher in subjects with asthma as compared with healthy subjects ($p < 0.001$).

RESULTS

Subjects

Sixty-two subjects were recruited. This study population comprised 34 individuals with asthma and 28 healthy individuals. The demographic, baseline lung function, and airway response data from these subjects are presented in Table 1.

Offline Measurements

Effect of flow rate. All 34 individuals with asthma and 28 healthy subjects performed exhaled gas collections for offline NO determinations at five expiratory flow rates as described. At each flow rate, individuals with asthma had higher FENO values (in part per billion ± SEM) as compared with healthy subjects: 57.9 ± 6.5 versus 26.3 ± 2.2; 34.9 ± 3.7 versus 16.7 ± 1.3; 23.0 ± 2.5 versus 11.4 ± 0.9; 17.5 ± 1.7 versus 8.8 ± 0.7; and 14.0 ± 1.2 versus 7.5 ± 0.6 at 50, 100, 200, 350, and 500 ml/second, respectively ($p < 0.001$ for comparisons at each flow rate, Figure 1). Offline FENO values fell with increasing flow rate; the mean FENO values at 100, 200, 350, and 500 ml/second were significantly lower that those at the next lowest flow rate for both subjects with asthma and normal subjects ($p < 0.0001$, Figure 1).

ROC analysis. ROC curves were constructed by plotting the sensitivity of offline FENO as a diagnostic test for asthma versus 1-specificity at each flow rate. The area under the ROC curves (AUCs), a measure of diagnostic power, ranged from 0.78 ± 0.06 to 0.82 ± 0.06. The discrimination between subjects with asthma and healthy subjects was robust at all flow rates ($p < 0.0001$, Table 2). This robust discriminatory power between individuals with asthma and healthy individuals was similar at low offline flow rates as compared with that at intermediate and higher offline expiratory flow rates ($p > 0.43$ for all comparisons).

TABLE 2. ROCs OF FENO AS A TEST FOR ASTHMA USING ONLINE AND OFFLINE TECHNIQUES AT MULTIPLE EXPIRATORY FLOW RATES

Technique	Flow Rate (*ml/s*)	AUC
Offline		
	50	0.79 ± 0.06
	100	0.79 ± 0.06
	200	0.81 ± 0.06
	350	0.82 ± 0.05
	500	0.82 ± 0.05
Online		
	42	0.84 ± 0.07
	108	0.86 ± 0.06
	210	0.82 ± 0.07
	250	0.83 ± 0.07

Definition of abbreviations: AUC = area under the receiver operating characteriastic curve; ROC = receiver operating characteristic.

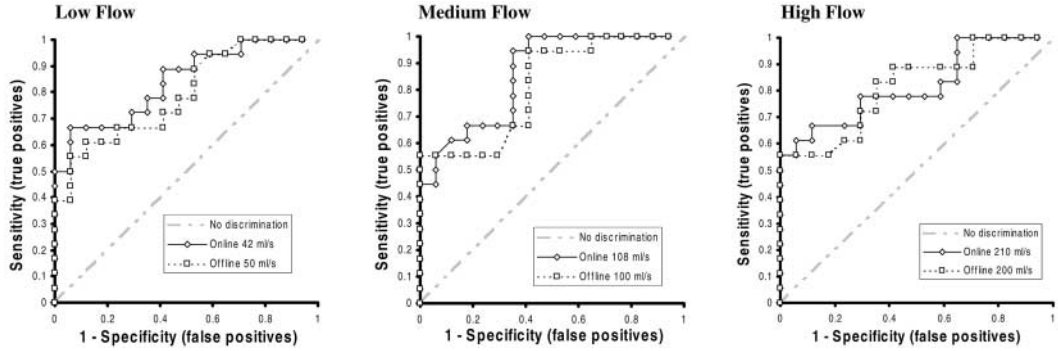

Figure 2. ROC curves for FeNO collected using online (*solid line*) and offline (*dashed line*) techniques. Over the range of flows specified in current ATS guidelines (low flow, 50 ml/s; medium flow, 100 ml/s; and high flow, 200 ml/s), the two techniques had a similar ability to discriminate healthy subjects from those with asthma ($p = 0.25$–0.98).

Online Measurements

Effect of flow rate. A subgroup of 18 subjects with asthma and 17 healthy subjects performed additional exhaled gas collections for online NO determinations at four expiratory flow rates as described. Similar to the offline measurements, individuals with asthma had higher mean FeNO values (in part per billion ± SEM) at each flow rate as compared with healthy subjects: 59.7 ± 8.6 versus 25.8 ± 2.6; 25.7 ± 3.4 versus 12.6 ± 1.2; 16.4 ± 1.9 versus 8.7 ± 0.6; 16.0 ± 2.0 versus 8.4 ± 0.7 at 42, 110, 210, and 250 ml/second, respectively ($p < 0.001$ for comparisons at each flow rate, Figure 1). Online FeNO values fell with increasing expiratory flow rate. The mean FeNO values at 108 and 210 ml/second were significantly lower than those at the next lowest flow rate for both subjects with asthma and healthy subjects ($p < 0.0001$). The smaller increase in flow from 210 to 250 ml/second did not produce significant changes in mean FeNO values in individuals with asthma and normal individuals ($p = 0.58$ and $p = 0.27$, respectively, Figure 1).

ROC analysis. ROC curves constructed for online measurements demonstrated that the AUC was similar at low flow rates as compared with that at intermediate and higher flow expiratory flow rates (Table 2). The AUC ranged from 0.82 ± 0.07 to 0.86 ± 0.06 (p for discrimination < 0.0001 at all flow rates, Table 2). The discrimination between individuals with asthma and healthy individuals was similar at low online flow rates as compared with that at intermediate and higher online expiratory flow rates. The AUC was 0.84 ± 0.07 at 42 ml/second, as compared with 0.86 ± 0.6 and 0.83 ± 0.07 at 108 and 250 ml/second, respectively ($p > 0.05$).

Offline versus online comparisons. To examine the diagnostic capability of offline FeNO measurements for asthma in comparison to online measurements at multiple flow rates, ROC curves generated from online and offline measurements in the same subjects were compared. At similar flow rates, the AUC derived from offline measurements was not statistically different from that derived from online measurements ($p = 0.25$–0.98, Figure 2). No combined effect of measurement technique and flow rate was noted. To exclude the possibility that subtle differences in discriminatory power between online and offline techniques could be magnified by extremes of flow, we compared the AUC derived from the fastest offline measurements with that obtained from the slowest online measurements and found no difference (0.80 ± 0.06 versus 0.84 ± 0.07, $p = 0.45$, Figure 3).

Sensitivity, specificity, and positive and negative predictive values for FeNO as a diagnostic test for asthma, at a cutoff of 1.96 SE above the mean healthy FeNO value at each of the flow rates, are presented in Table 3. The sensitivity and specificity for a diagnosis of asthma were generally 70% or greater for both techniques and all flow rates. The positive and negative predictive values are calculated using both the prevalence of asthma in the study group (0.55) and in the general population (0.05). These measures of diagnostic power were generally similar using both online and offline collections over the range of flow rates studied.

DISCUSSION

FeNO is characteristically elevated in asthma and has been demonstrated to distinguish individuals without asthma from individuals with asthma in several reports (1, 9, 24, 25). However, controversy exists regarding which flow rate and measurement technique should be used to distinguish optimally patients with asthma from healthy subjects (12–15, 22, 26). In this study, we investigated the effect of expiratory flow rate and measurement technique on the ability of FeNO to detect subjects with and without asthma in a well-characterized study population. We found that the diagnostic power of FeNO for asthma is not influenced by either the expiratory flow rate across the range of flows in current use or by measurement technique (online or offline).

To examine the influence of flow rate and collection method on the ability of FeNO to discriminate between subjects with asthma and healthy subjects, we constructed ROC curves for a diagnosis of asthma and then compared the AUC constructed

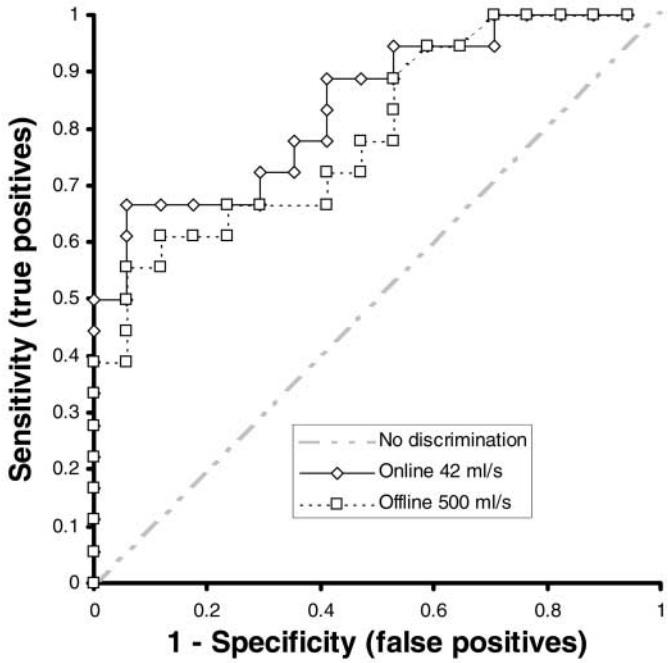

Figure 3. ROC curves for FeNO collected using a slow exhalation online (*solid line*) and fast exhalation offline (*dashed line*) technique. The discriminatory power of the slowest online technique was not different from that of the fastest offline technique ($p = 0.45$).

TABLE 3. PERFORMANCE CHARACTERISTICS OF FENO AS A TEST FOR ASTHMA

Technique	Flow Rate (*ml/s*)	Cutoff Value (*parts per billion*)*	Sensitivity	Specificity	Study Group† PPV	Study Group† NPV	General Population‡ PPV	General Population‡ NPV
Offline								
	50	30.7	70.6	75.0	74.6	71.0	12.9	98.0
	100	19.2	64.7	67.7	65.9	59.4	8.0	97.0
	200	13.2	73.5	71.4	75.1	69.7	11.9	98.1
	350	10.4	79.4	71.4	76.5	74.7	12.8	98.5
	500	8.7	66.7	71.4	73.3	64.6	10.9	97.6
Online								
	42	30.9	72.2	70.6	71.9	70.9	11.4	98
	108	14.4	66.7	70.6	70.2	67.0	10.7	97.6
	210	10.0	66.7	70.6	70.2	67.0	10.7	97.6
	250	9.9	72.2	76.5	76.2	72.6	13.9	98.1

Definition of abbreviations: FENO = fraction of exhaled nitric oxide; NPV = negative predictive value; PPV = positive predictive value.
* Cutoff values are 1.96 × SE above the mean healthy value at that flow rate.
† Asthma prevalence in the study group = 0.55.
‡ Asthma prevalence in the general population = 0.05.

using the data obtained from these subjects at multiple flow rates using online and offline methods. ROC curves plot the sensitivity (true positive rate) of a test against 1-specificity (false-negative rate) of the test at all possible thresholds. The curve developed by such a plot is a graphic representation of the trade-off between sensitivity and specificity of the test, and the AUC is a measure of the power of the test to discriminate between the presence and absence of the condition being studied. The relative discriminatory power of two tests can be determined by comparing the AUC using established statistical techniques (23). We felt that this analytic approach was indicated because although significance levels (p values) for differences in mean values indicate how likely such observed differences would be expected to occur by chance, they do not quantify discriminatory capacity.

Using ROC analysis, we have documented that the diagnostic power of FENO for asthma is robust. The AUC observed with all flow rates and both techniques studied varied over a narrow range from 0.78 to 0.84; these values are comparable to that for serum creatine kinase MB fraction measured at 6 hours after the onset of chest pain to predict myocardial infarction (AUC = 0.778) (27). Within our study population (prevalence of asthma = 0.55), this discriminatory capacity produced a positive and negative predictive value for asthma of approximately 70% (Table 3). It is important to note that extrapolation of our findings to the general population (prevalence of asthma = 0.05) indicates that FENO as a test for asthma would demonstrate a positive predictive value of approximately 12% with a negative predictive value of 97 to 98% (Table 3). These observations suggest that FENO is most likely to be useful as an exclusionary test for asthma.

Other investigators have interpreted the currently available data regarding flow dependence of NO recordings to imply that measurements made at low flow rates "amplify the NO signal and provide better discrimination between health and disease states" (14). However, the effect of flow rate on discrimination has not been rigorously investigated. Our results, which demonstrate highly significant elevation in FENO in subjects with asthma as compared with normal subjects at each flow rate, indicate that despite flow dependence, the distinction between subjects with asthma and healthy individuals is intact at all flow rates studied. These findings are consistent with those recently published by Delclaux and colleagues that demonstrate grossly similar discrimination between individuals with asthma and healthy individuals using online NO collections at 50 ml/second as compared with those collected at 200 ml/second (28). Furthermore, we did not detect differences between either the AUC obtained using different flow rates and the same collection method (online or offline) or that obtained at similar flow rates in comparison to the alternate collection method. Thus, our analysis indicates that although the numerical differences in FENO between individuals with asthma and healthy individuals may be smaller at higher flow rates, the discriminatory power is preserved regardless of which flow or measurement technique is used. This may be due to the close correlation between online and offline techniques as well as to the decrease in the variability around the mean values observed with increased flows coupled with the sensitivity of current analyzers of approximately 0.5–1.0 parts per billion (13). Alternatively, it is possible that a difference in diagnostic power does exist and was not detected in this study. Although it is possible that a β-type error may have occurred, we think this possibility is unlikely given the largely overlapping standard errors for the AUCs (Table 2).

Our data additionally demonstrate that recorded FENO values are inversely related to expiratory flow rate for measurements made using both online and offline techniques. These data extend and confirm those previously reported by others. Specifically, Silkoff and colleagues studied subjects without asthma and found that using online techniques, FENO increased 35-fold as expiratory flow decreased from 1,550 to 4.2 ml/second (12). We studied both individuals with asthma and healthy individuals with offline methods over the narrower range of flow rates described in recent guidelines for FENO measurement, and we documented a 3.5- to 4.5-fold increase in FENO as flow rates decreased from 500 to 50 ml/second (22). Although this increment in FENO with lower flow rates is less than that reported by Silkoff and associates, our finding is not unexpected; the largest increases in FENO in their study occurred at very low flow rates not currently used for routine NO collection and not included in our report (i.e., < 50 ml/second). Inspection of their published online data indicates that a 3- to 4-fold increase in FENO occurred over the flow range of 500–50 ml/second, similar to our findings using offline techniques.

Our findings also extend those of Kissoon and colleagues, who compared online and offline collections in healthy adolescents made at very low flow rates (4–46 ml/second) (14). We document offline flow dependence over the range of flows suggested in current ATS guidelines as well as demonstrate that the effect occurs in individuals with and without asthma

regardless of the measurement technique used. This observation is important because measurement of FENO is currently being commercially marketed as a method for clinically monitoring patients with asthma, and no studies to date have systematically examined the interaction of flow dependence and measurement technique in this population.

In summary, we have documented in a systematic manner that measurement of expired NO is a robust discriminator between healthy subjects and subjects with asthma and that this discriminatory capacity is not dependent on flow rate or on which of the ATS-specified measurement methods (online or offline) are used. Furthermore, we have demonstrated that the dependence of measured FENO on expiratory flow rates occurs across the range of flows specified in current guidelines, that this dependence occurs in healthy individuals and individuals with asthma and is not restricted to measurements made with online techniques. In light of these findings, investigators and clinicians, while rigorously controlling flow during the collection maneuver and other factors outlined in the ATS guidelines (i.e., smoking status, recent upper respiratory infections), may choose a flow rate (within the range reported here) that is most comfortable and convenient for the population being studied (22). Because FENO is very sensitive to changes in expiratory flow rate, once selected, the same flow rate must be used for repeated measurements within the same individual and for legitimate comparisons between individuals. If FENO determinations are to be used in screening for asthma or monitoring of patients undergoing treatment, our results suggest that offline measurements, which provide maximally efficient use of the analyzer, can be employed without a decrement in discriminatory power.

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