CLINICAL STUDY

A purification step prior to commercial sensitive immunoassay is necessary to achieve clinical usefulness when quantifying serum 17 β -estradiol in prepubertal children

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Abstract

Objective: To test the clinical usefulness of sensitive commercial immunoassays for determination of low 17 β -estradiol concentrations in children.

Methods: The lower limit of detection and clinical usefulness (functional sensitivity) of three commercial estradiol immunoassays were validated by use of 500 sera from prepubertal and pubertal children and 55 pooled sera. The three immunoassays consisted of two modified direct immunoassays; one RIA (Spectria Estradiol RIA) and one time-resolved fluoroimmunoassay (AutoDELFIA Estradiol), both with increased serum volume in relation to antibody concentration and extended incubation time. In the third method, serum was purified and concentrated using diethyl ether extraction prior to measurement by the modified Spectria Estradiol RIA.

Results: The lower limits of detection and clinical usefulness were 9 and 30 pmol/l for the direct RIA, 11 and 50 pmol/l for the AutoDELFIA, and 4 and 6 pmol/l for serum determined by extraction RIA. When measuring the serum pool originating from girls at breast stages 1–2, the direct RIA and AutoDELFIA resulted in significantly higher 17 β -estradiol concentrations when compared with the extraction RIA (+58 and +267%, $P < 0.001$). We found a significant difference in 17 β -estradiol concentrations between girls at breast stages 1 (median 6 pmol/l) and 2 (median 16 pmol/l), when quantified by the extraction RIA ($P < 0.0001$) but no difference when quantified with the direct RIA (median values 12 and 14 pmol/l respectively).

Conclusion: For determination of low serum 17 β -estradiol concentrations in children, an extraction step prior to commercial immunoassay is needed to achieve clinically useful results.

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Introduction

Specific and sensitive assays for 17 β -estradiol measurements are needed in clinical practice for evaluation of pubertal disorders in children and low postmenopausal levels in women, as well as for monitoring aromatase inhibitor therapy and sex replacement hormone therapy in both adults and children.

Measurements of 17 β -estradiol obtained by mass spectrometry identification are considered as the reference method (1–3). However, until mass spectrometry is practical for widespread use, the most widely used technique for the measurement of serum 17 β -estradiol is and will be immunoassays, due to its simplicity and reliability. A trend is to use high-performance automated instruments with use of small specimen volumes. For the most part, these commercial assays are designed for fertility investigations in female adults, but they are not sensitive enough for quantifying the low levels present in children (4), men, and postmenopausal women (3, 5). Despite improvements regarding sensitivity during recent

years, direct assays have not been thoroughly validated for low 17 β -estradiol concentrations in serum and have not reached the degree of sensitivity and specificity in clinical practice as expected from the manufacturers' documentation. It has been shown that in samples from postmenopausal women with low 17 β -estradiol, extraction RIAs correlate better with mass spectrometry than direct assays do (3).

In this study, we evaluate two sensitive commercial 17 β -estradiol immunoassays for clinical usefulness in prepubertal and pubertal children. As our standard for comparison, we use an extraction RIA which we have used in our clinical practice for a decade (6).

Materials and methods

Clinical samples

All samples used in this study are serum samples submitted for routine clinical testing in children. After

17 β -estradiol measurements for routine clinical testing, identification markings were removed from all samples. In the comparison between the direct RIA and extraction RIA, pubertal stages according to Tanner (7) and ages were registered in addition to 17 β -estradiol values.

Assay procedure

Spectria direct RIA The original assay was a coated tube RIA (Spectria Estradiol RIA, Orion Diagnostica, Espoo, Finland) with lower detection limit 30 pmol/l (defined as the apparent concentration two s.d. from the counts at maximum binding). The cross-reactivity of the estradiol antiserum had been tested by the manufacture for 30 estradiol-related steroids. The cross-reactivity was below 1% for estrone, 16-oxestradiol, estradiol-3-glucuronide, estriol, and 16-hydroxy estrone, below 0.1% for progesterone, corticosterone, 2-hydroxyestradiol, and below 0.001% for the rest of the related steroids.

To increase the sensitivity of the RIA, 150 μ l serum was used instead of 100 μ l. The incubation time was extended from 2 h at 37 °C to 16–19 h at room temperature. In addition to the kit calibrators with the lowest concentration of 50 pmol/l, three calibrators of lower concentrations (6.25, 12.5, and 25 pmol/l) were prepared by dilution of the 50 pmol/l calibrator with the zero calibrator. Otherwise, the RIA was conducted according to the manufacturer's instructions.

Spectria extraction RIA In the extraction RIA, serum was purified and concentrated using diethyl ether extraction prior to quantifying the 17 β -estradiol concentrations by Spectria. The basic method has been published previously (6, 8), although the latest revision has not been published before. In the present study, 4 ml diethyl ether (high performance liquid chromatography grade, Merck 1.00921) was added by a dispenser (Hirschmann Laborgeräte, Eberstadt, Germany) to 450 μ l serum in 10 ml glass tubes with a tight stopper (rubber, coated with Teflon). For confirmation of the detection limit when the aim was to only purify the serum, 2 ml diethyl ether was added to 200 μ l serum in 10 ml glass tubes. The sex steroids were extracted to the ether phase for 30 min in a rotation mixer. The serum phase was thereafter frozen by putting the tubes in a beaker with a mixture of 99.5% ethanol and dried ice. The ether phase was transferred to 5 ml glass tubes, and totally evaporated on a 37 °C heat block under a stream of nitrogen. Reconstitution was made using 200 μ l zero calibrator (Spectria Estradiol zero calibrator, Orion Diagnostica). After incubation at 37 °C for 20 min and vortexing, the samples were ready for quantification by the modified Spectria Estradiol RIA, using 150 μ l. The entire extraction procedure was done in duplicate.

To assess recovery of the extraction procedure, serum samples from ten different children were incubated with a small amount of tritiated 17 β -estradiol for 2–3 h in a shaking water bath (37 °C). The extraction procedure was then performed as above. The radioactivity was determined in 150 μ l of the final sample volume. The recovery of 17 β -estradiol using the extraction procedure was $91 \pm 1\%$ (mean $\pm$ s.d.), which was close to the theoretical recovery of 90%. These results are in line with our earlier estimation of the extraction procedure (6). All results have been corrected for the extraction loss.

AutoDELFIA assay The original assay is an automated time-resolved fluoroimmunoassay (AutoDELFIA Estradiol, Wallac oy, Turku, Finland) with a lower detection limit 50 pmol/l, as stated by the manufacturer and Brito *et al.* (9). Required sample volume is 25 μ l. To increase the sensitivity of the assay, the antibody was diluted 1:18 instead of 1:10 and incubation time was extended to 2 h. In addition to the kit calibrators; with the lowest concentration of 50 pmol/l, one calibrator of lower concentrations (15 pmol/l) was prepared by dilution with a zero calibrator. Otherwise, the AutoDELFIA was conducted according to the manufacturer's instructions.

Assay evaluation

Spectria assay To estimate the lower analytical detection limit, replicates of 20 zero calibrators were measured in three different assays of different kit lots. The analytical sensitivity was confirmed in dilution tests in which 100 serum samples from children were purified by the extraction procedure using 200 μ l serum and 2 ml diethyl ether, and thereafter diluted 1:2 with the zero calibrator from the kit. In addition, two purified serum samples were serially diluted 1:2 with the zero calibrator down to the estimated detection limit.

Assay precision was assessed by use of pooled serum from children and one commercial quality control (Lyphochek from Bio-Rad). Each serum pool was measured as a duplicate in 10–20 different assays of at least four different kit lots. The interassay coefficient of variations (CV) were calculated from the mean of the duplicates. Clinical usefulness (usually termed functional sensitivity) was defined as the lowest concentration of an assay that can be measured with an interassay CV of 20%.

To assess accuracy, 17 β -estradiol was removed by charcoal stripping in two different pooled child sera, originating from prepubertal children and children in early puberty. After charcoal stripping, the samples were divided in two, and 24 pmol/l 17 β -estradiol was added (spiked) in one of each pair of charcoal stripped child sera.

Charcoal stripped sera and spiked sera were thereafter measured with direct RIA and extraction RIA.

The concentration of sex hormone-binding globulin (SHBG) in the zero calibrator was determined in four different zero calibrator lots by RIA (Orion Diagnostica).

To test the clinical reliability of the extraction RIA, all 17 β -estradiol results during the years 2004–2006 originated from girls with precocious puberty undergoing long-acting gonadotrophin-releasing hormone (GnRH) analog treatment. The goal of the treatment was to reversibly stop pubertal development by suppressing gonadotropins/17 β -estradiol levels to the prepubertal range.

The AutoDELFIA assay The minimal detection concentration for the modified sensitive AutoDELFIA was calculated with the aid of a precision profile, defined as the lowest level that could be measured with an intra-assay CV of 10%. Assay precision was tested in four child serum pools: girls breast stages 1–2, girls breast stage 2, girls breast stages 2–4 (7), and one commercial control.

Comparison between extraction RIA and direct immunoassays

To evaluate the concordance between the direct immunoassays and the established extraction RIA, 275 serum samples were run in parallel with the direct RIA and the extraction RIA. The serum samples originated from 229 females aged 0–19 years (median 11 years) at pubertal stages 1–5 and 46 pooled child sera. In addition, 122 children's serum samples were

run in parallel with the AutoDELFIA and the extraction RIA.

Statistical analysis

Linear regression analyses were performed on log-transformed data. Method comparison was evaluated by a Bland–Altman plot (10). Undetectable values, i.e., values below zero, were set to 1 pmol/l. A value of $P < 0.05$ was considered significant (11).

Results

Assay validations

The modified Spectria's analytical detection limit was 9 pmol/l, calculated as the apparent concentration 3s.d. from the counts at maximum binding. The analytical detection limit was confirmed in dilution tests, in which purified, not concentrated, serum samples from children could be diluted down to 9 pmol/l and obtain expected 17 β -estradiol concentrations (Fig. 1A). The purified samples showed linearity down to the detection limit of 9 pmol/l and even lower concentrations (Fig. 1B). An analytical detection limit of 9 pmol/l is equal to 4 pmol/l (1.2 pg/ml) for serum samples that have been concentrated in the diethyl ether extraction step prior to quantification by Spectria Estradiol RIA.

The minimal detection concentration for the modified sensitive AutoDELFIA was estimated to be 11 pmol/l by the automated instrument.

The results of imprecision studies conducted with child serum pools are summarized in Table 1. The serum pool originating from girls at breast stages 1–2,

Figure 1 Confirmation of the analytical detection limit for the modified 17 β -estradiol RIA by dilution tests. (A) One hundred child serum samples were purified from potentially interfering substances by the diethyl ether extraction step. Each of these was diluted 1:2 using zero calibrator from the Spectria Estradiol kit. Undiluted and diluted serum samples were measured in duplicate by the modified RIA. Serum 17 β -estradiol concentrations could be diluted down to 9 pmol/l (the estimated lower limit of detection for the RIA) and provide an adequate reading. (B) Linear regression analyses between expected and observed 17 β -estradiol concentrations after serial dilution in two samples where the steroids had been purified by diethyl ether extraction and thereafter reconstituted in zero calibrator before serial dilution, maintaining a constant SHBG concentration. Linear regression analyses are calculated on the filled symbols.

Table 1 Summary of interassay coefficient of variations (CVs) for pooled child samples measured by extraction RIA (extraction + modified Spectria Estradiol RIA, Orion Diagnostica, Espoo, Finland), direct RIA (modified Spectria Estradiol RIA, Orion Diagnostica) and AutoDELFIA (modified automated time-resolved fluoroimmunoassay, Wallac Oy, Turku, Finland). Each serum pool was measured as a duplicate in 5–20 different assays. The interassay CVs were calculated from the mean of the duplicates. Pubertal breast development was assessed according to Tanner (7).

Origin of serum pool	Extraction RIA			Direct RIA			AutoDELFIA		
	n	Mean 17 β -estradiol (pmol/l)	CV (%)	n	Mean 17 β -estradiol (pmol/l)	CV (%)	n	Mean 17 β -estradiol (pmol/l)	CV (%)
1. Prepubertal children	20	4	40	–	–	–	–	–	–
2. Prepubertal children	10	6	19	10	17	38	–	–	–
3. Prepubertal girls and girls at breast stage 2	20	12	14	20	19	12	5	44	16
4. Girls at breast stage 2	20	23	11	–	–	–	5	55	14
5. Girls at breast stages 2 and 3	20	37	9	20	42	13	–	–	–
6. Girls at breast stages 2–4	–	–	–	20	71	8	5	104	11
7. Lyphochek 40161	–	–	–	20	241	5	5	313	5

resulted in significantly higher 17 β -estradiol concentrations when measured by the direct RIA and AutoDELFIA when compared with measurements by the extraction Spectria ($n=20$, $P<0.001$, +58% and $n=5$, $P<0.001$, +267% respectively).

Charcoal stripping of child sera resulted in mean 17 β -estradiol concentration below the detection limit (<4 pmol/l) when determined by extraction RIA but resulted in a mean value of 15 pmol/l when determined by direct RIA. The corresponding spiked sera resulted in concentration of 22 pmol/l when determined by extraction RIA and 35 pmol/l when determined by direct RIA. The expected concentration in the spiked sera was 24 pmol/l.

Our laboratory participates in the Bio-Rad External Quality Assurance Services. For the year 2006, the direct 17 β -estradiol RIA's and the extraction 17 β -estradiol RIA's bias from the mean of 125 laboratory results were –24 and –26% respectively.

The SHBG concentrations in the zero calibrators range between 10 and 26 nmol/l (median 25 nmol/l).

Clinical usefulness

The functional sensitivity of the concentration where the interassay CV is 20% is estimated at 6 pmol/l (1.6 pg/ml) for the extraction RIA (Table 1). The 17 β -estradiol concentrations quantified with the extraction RIA in samples from girls (aged 4–17 years) during prepuberty (with Tanner breast stage 1; $n=47$) and early puberty (Tanner breast stage 2; $n=54$) ranged between <4–25 and 5–88 pmol/l, with median values 6 and 16 pmol/l respectively. The corresponding values for the direct RIA was <9–46 and <9–70 pmol/l, with median values 12 and 14 respectively. We found a significant difference in 17 β -estradiol concentrations between girls with breast stages 1 (prepubertal) and 2 (early puberty), when quantified by the extraction RIA ($P<0.0001$) but no difference when quantified with the direct RIA. Samples from girls with breast stage 1, measured by the direct RIA, were significantly higher than those measured by the extraction RIA ($P<0.01$).

The clinical reliability of the extraction RIA is demonstrated in Fig. 2. In total, 37 serum samples from girls with precocious puberty had been quantified for 17 β -estradiol measurements at our laboratory. Seven of these samples were submitted due to clinical suspicion of lack of treatment efficacy (pubertal progress). All these samples resulted in 17 β -estradiol concentrations in the pubertal range. Thirty samples were sent as a routine control. Out of 30 cases, 24 resulted in levels seen during prepuberty.

The functional sensitivity of the concentration where the interassay CV is 20% is estimated to be between 17 and 42 pmol/l for the direct RIA (Table 1). The results from the relationship between samples purified and concentrated by extraction with diethyl ether prior to

Figure 2 Serum 17 β -estradiol concentrations in girls with precocious puberty during long-acting GnRH analog treatment when compared with spontaneous 17 β -estradiol secretions in healthy prepubertal girls. The gray area represents peak value of 17 β -estradiol levels seen in healthy prepubertal girls. During the years 2004–2006, in total 37 samples were analyzed for therapy check-up during long-acting GnRH analog treatment. In seven cases, the reason for sending the samples was clinical suspicion of lack of treatment efficacy (filled circles), suspected to have 17 β -estradiol in the pubertal range and in the other 30 cases there was no more information than 'check-up of treatment' (open circles).

measurement by RIA and samples quantified directly by RIA are shown in Fig. 3. We found a close correlation for 17 β -estradiol concentrations down to the estimated analytical detection limit for the Spectria, 9 pmol/l ($r=0.93$, $P<0.0001$, $n=219$, $Y=0.95X+1.78$, Fig. 3A). However, below 30 pmol/l, the discrepancy between the two methods is far above 20% (Fig. 3B). The results for the linear regression analysis of 17 β -estradiol concentrations, above 30 pmol/l, were: $r=0.97$, $P<0.0001$, $n=94$, $Y=0.97X-0.92$. Therefore, we establish the clinical usefulness to be 30 pmol/l for the direct RIA.

The functional sensitivity of the concentration where the interassay CV is 20% is estimated to be below 44 pmol/l for the AutoDELFIA (Table 1). The results from the relationship between samples quantified directly by AutoDELFIA and samples quantified by the extraction RIA are shown in Fig. 4. We found a close correlation for 17 β -estradiol concentrations between the two assays ($r=0.92$, $P<0.0001$, $n=108$, $Y=0.98X+17.5$, Fig. 4A) for values above the analytical detection limits. However, below 50 pmol/l, the discrepancy between the two methods was far above 20% (Fig. 4B). Therefore, we establish the clinical usefulness to be 50 pmol/l for the AutoDELFIA. The AutoDELFIA method's difference from the mean value of extraction RIA and AutoDELFIA resulted in 20% higher 17 β -estradiol concentrations (Fig. 4B).

Discussion

We present three 17 β -estradiol immunoassays with low analytical detection limits (4, 9, and 11 pmol/l). However, only the extraction RIA obtained a functional sensitivity close to the analytical sensitivity. In addition, the extraction RIA manages to distinguish prepubertal values from early pubertal values. While the extraction RIA gives clinically useful results down to 6 pmol/l, the direct immunoassays could only perform clinically useful results down to 30–50 pmol/l.

Previously published papers have shown the importance of using an extraction step prior to immunoassay for determination of the low serum concentration present in postmenopausal women (3, 5). In our study, we show the importance of a preceding purification and concentration of the analyte prior to quantification by commercial RIA for determination of low serum 17 β -estradiol concentrations in children. Together, these studies establish that direct immunoassays do not perform well at low 17 β -estradiol concentrations, not even those with low analytical detection limit. This may be due to potentially interfering substances, particularly cross-reacting water-soluble steroid conjugates, binding proteins (especially SHBG), and non-specific binding (NSB). Concentrations of SHBG are known to be high in prepubertal children (median 92 nmol/l) (12, 13) and postmenopausal women (mean 103 nmol/l) (14), and in combination with the 5000–15 000 times lower 17 β -estradiol concentrations it is a challenge to quantify accurate concentrations of 17 β -estradiol. In this study, we show that the 17 β -estradiol calibrators included in the Spectria kit on average contain four times lower SHBG concentrations when compared with that in children's sera. To obtain accurate results with a direct immunoassay, the concentration of SHBG, and NSB in a patient's serum samples ought to be similar to that in the calibrators (15–17). Using an extraction step prior to quantification by RIA, the patient's SHBG and NSB are replaced by a zero calibrator containing the same concentration of SHBG and NSB as is found in the calibrators in the kit. However, if the assay has problems with high cross-reactivity, an extraction does not overcome these problems. The Spectria assay that we use has very low cross-reactivity with estradiol-related steroids.

By charcoal stripping, it was confirmed that the interfering substances present in child sera are removed by the ether extraction procedure. Charcoal stripping removes 99% of initially present estradiol (18) and is a common procedure when producing zero calibrators. In this study, the expected 17 β -estradiol concentration after charcoal stripping was 0 pmol/l and after spiking, the expected 17 β -estradiol concentration was 24 pmol/l. The extraction RIA resulted in correct 17 β -estradiol concentrations, both before and after spiking, but the direct RIA

Figure 3 Assay comparison between the extraction RIA and the direct RIA. (A) Correlation in 17β-estradiol concentrations between samples that have been purified by extraction of diethyl ether preceding RIA and serum quantified directly by RIA ($n=275$). Linear regression analysis (filled symbols) excludes values below the detection limits ($r=0.93$, $n=219$, $P<0.0001$). (B) Bland-Altman plot of the difference in 17β-estradiol concentrations (serum quantified directly in the RIA minus samples that have been extracted by diethyl ether preceding RIA expressed as a percentage of the mean) against the mean of direct RIA and extraction RIA.

Figure 4 Assay comparison between the extraction RIA and direct AutoDELFIA. (A) Correlation in 17β-estradiol concentrations between samples that have been purified by extraction of diethyl ether preceding RIA and serum quantified directly by AutoDELFIA ($n=122$). Linear regression analysis (filled symbols) excludes values below the detection limits ($r=0.92$, $n=108$, $P<0.0001$). (B) Bland-Altman plot of the difference in 17β-estradiol concentrations (serum quantified by AutoDELFIA minus samples quantified by extraction RIA expressed as a percentage of the mean) against the mean of AutoDELFIA and extraction RIA.

resulted in falsely high concentrations both after charcoal stripping and after addition of known amounts of 17β-estradiol.

The overestimation of 17β-estradiol concentrations in the direct immunoassays was also demonstrated by the impression data. The pooled serum samples originating from prepubertal girls and girls at breast stage 2 generally resulted in an overestimation. It is typical that the serum pools numbered 2 and 3 resulted in similar mean 17β-estradiol concentrations when measured by the direct RIA, but the serum pool originating from prepubertal children resulted in a much higher interassay CV than the serum pool originating from both prepubertal and pubertal girls. The results obtained by the AutoDELFIA

are still overestimating and are similar to those obtained by the original method (9).

By directly or indirectly increasing the sample volumes and incubation times, we decreased the analytical detection limits from 30 and 50 pmol/l down to 9 and 11 pmol/l respectively, but we did not improve the clinical usefulness. The analytical sensitivity is estimated by determining the imprecision of the zero calibrator, which does not always correspond to the detection limit in the intended specimen. This is because the specimens are less pure and may contain interfering substances. In this study, purification by diethyl ether extraction of the children's serum samples was required to confirm the analytical sensitivity.

The most sensitive assays are considered to be the recombinant cell bioassays (RCBA). Klein and co-workers have developed a RCBA to measure estrogenic activity with a detection limit between 0.07 and 0.7 pmol/l 17 β -estradiol equivalents. The sample volume used was 800 μ l, the interassay CV was 13% at 7 pmol/l and increased to about 60% at 0.07 pmol/l (19, 20). Another RCBA has been developed by Paris *et al.* (21). The required sample volume is 800 μ l and the detection limit is below 4 pmol/l. The interassay CV was 20% at 6 pmol/l. It has been shown previously that RCBA correlates to gas chromatography–tandem mass spectrometry (22). The extraction RIA corresponds well with RCBA for values down to the analytical limits (23). Furthermore, the 17 β -estradiol values obtained in prepubertal girls with our extraction RIA are more or less identical to those obtained by RCBA (20, 21). By compilation of all this data, we estimate that our modified commercial extraction RIA has similar clinical usefulness as RCBAs.

There are three clinical situations in pediatrics where there is a need to determine low 17 β -estradiol levels in girls to: 1) establish whether puberty has started, 2) monitor GnRH (lutening hormone releasing hormone) analog treatment of precocious puberty and 3) monitor hormone replacement therapy in hypogonadal children. The extraction RIA has the sensitivity to establish whether puberty has started or not and to determine the low 17 β -estradiol levels seen at pubertal induction with transdermal estradiol patches in hypogonadal girls (6, 8). Figure 2 shows the results from 37 samples sent to our laboratory during the years 2004–2006 for therapy control of treatment of precocious puberty with long-acting GnRH analog. The goal of the treatment is to reversibly stop pubertal development, by suppressing gonadotropins/17 β -estradiol levels to the prepubertal range (24). The state of the art method is to show that the efficacy of the treatment has been accomplished by GnRH challenge test. However, development of sensitive methods for determining 17 β -estradiol makes it possible to assess the efficacy of the treatment more simply and inexpensively by determination of 17 β -estradiol instead of gonadotropins after GnRH test (25). Some pediatricians have used this possibility and sent samples to our laboratory for analysis. In all seven cases where the reason for analysis was clinical suspicion of lack of efficacy, all the samples had pubertal levels. In 24 out of 30 cases where there was no information about the efficacy, serum 17 β -estradiol concentrations were in the prepubertal range or under the detection limit of the assay. Even if the result is further confounded by not knowing the reasons for therapy control, the result lends further support to the theory that the assay works for use in a clinical setting. The extraction RIA is at present an accredited assay by SWEDAC in Sweden, SS-EN ISO 15189 (no. 1899).

In conclusion, commercially sensitive immunoassays do not give clinically useful results in sera from

prepubertal children and children in early puberty. A purification step prior to commercially sensitive immunoassay is necessary to achieve clinically useful results when quantifying serum 17 β -estradiol in children. Clinical laboratories should be aware that the clinical usefulness of their current procedures may be far from the method's analytical detection limit. It is important to validate each assay for its intended purpose.

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