Pantex
Division of Bio-Analysis, Inc.

Providing Diagnostic Technology for a Better Tomorrow

DIRECT SALIVARY 17ß-Estradiol

Enzyme Immunoassay Kit

For in-vitro diagnostic use (IVD)

Catalog Number: 674

IVD - FDA Registered

Rev 9 12/2020

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TABLE OF CONTENTS

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I. Intended Use and Description

The Pantex Direct Salivary 17β-Estradiol EIA Kit, Cat #674, is designed and validated for the quantitative measurement of 17ß-Estradiol in human saliva. Results obtained by this device may be used in the diagnosis and treatment of various hormonal sexual disorders. This test is not intended for assessing placental function in complicated pregnancy.

Warning:
Estradiol immunoassays have been reported to demonstrate significant cross-reactivity with the drug Fulvestrant (Faslodex^{R}). This cross-reactivity can cause falsely elevated estradiol levels in patients being treated with Fulvestrant. Due to the possibility of this cross-reactivity, the Pantex line of Estradiol Immunoassays should not be used for patients being treated with the drug Fulvestrant. Fulvestrant (Faslodex^{R}) is used to treat a certain type of breast cancer in postmenopausal women. The use of falsely elevated estradiol test results in these patients could lead a clinician to alter or discontinue the patient's therapy.

II. Assay Background

17ß-Estradiol (E2) is a steroid hormone produced mainly by the Graafian follicle of the ovary female and in small amounts by the testes in male subjects. E2 is biologically the most active of naturally produced human estrogens. The majority of E2 (98%) is bound to sex hormone binding globulin (SHBH) and to a lesser extent to other serum proteins such as albumin. Only a small fraction circulates in the free form or conjugated to sulfates and glucuronides (2,3). In non-pregnant women there is a cyclic variation in the concentration of E2, the highest values being measured usually the day before ovulation (4,5). Positive feedback influence of this peak value is considered essential for occurrence of the midcycle luteinizing hormone (hLH) peak and consequently, for ovulation (6). During pregnancy the E2 concentration increases considerably and remains high throughout pregnancy (7).

The assay of E2 is a valuable tool for assessing the etiology of amenorrhea and/or infertility in female subjects. It is also a useful aid in monitoring ovulation induction treatment with clomiphene citrate, LH-RH (LH-releasing hormone) or exogenous gonadotropins (8,9). In male subjects, serum E2 measurements are used for investigating feminizing syndromes (10).

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III. Assay Principle

The Pantex Direct Salivary 17β-Estradiol EIA Kit, Cat #674, is based on the competition principal and microplate separation. An unknown amount of estradiol present in a saliva sample and a fixed amount of estradiol conjugated to horse radish peroxidase (E2-HRP) compete for binding sites with a rabbit monoclonal estradiol antiserum bound to GARGG (goat anti-rabbit gamma globulin) coated wells of a microplate. After incubation, unbound components are washed away. Enzyme substrate solution is then added, and a blue color formed. This reaction is stopped with an acid solution to produce a yellow color. The optical density is then read at 450 nm. The amount of E2-HRP detected is inversely proportional to the amount of estradiol in a sample.

IV. Reagents Provided and Reagent Preparation

1. **GARGG Plate**: One 96 well microplate (12x8 breakable strip wells) coated with goat anti-rabbit gamma globulin (GARGG) placed in a resealable foil bag with desiccant. One (1) 96 well kit is sufficient for 78 singlicate or 39 duplicate patient measurements.
2. **Stock Estradiol (17-ßE2) in BSA buffer.**

   Concentration: 3200 pg/mL, 1 bottle, 0.250 mL Working calibrators preparation: From stock **3200 pg/mL**, dilute **1:100** with **assay buffer** to obtain the **32 pg/mL** calibrator. Make serial dilutions (**in assay buffer**) starting with the **32 pg/mL** calibrator to obtain the following calibrator concentrations: **16 pg/mL, 8 pg/mL**, **4 pg/mL, 2 pg/mL**, **1 pg/mL**. **"0" calibrator** is **assay buffer**.

   *Concentrations of 17-ß Estradiol calibrators are actual and traceable to US Pharmacopeia (USP) Cat No. 25000-8, Lot NoJ191
3. Assay buffer. 1 bottle, 20 mL.
4. Salivary Estradiol EIA Control #1. 1 bottle, 1.0 mL. Concentration is on the label and is traceable to Estradiol (17ß-Estradiol) from USP.
5. Salivary Estradiol EIA Control #2: 1 bottle, 1.0 mL. Concentration is on the label and is traceable to Estradiol (17ß-Estradiol) from USP.
6. Salivary **Estradiol EIA Antibody**. 1 bottle, 3 mL. The solution is blue.
7. Salivary **Estradiol Horse Radish Peroxidase Conjugate concentrate (E2-HRP concentrate**) 1 amber bottle, 0.200 mL. The solution is yellow and light sensitive.
8. E2-HRP **Conjugate Buffer.** 4 mL. To be used for **E2-HRP working reagent** preparation **ONLY**.

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E2-HRP working reagent preparation: Determine the amount of working **E2-HRP working reagent** needed and dilute 1:20 with **conjugate buffer**. For example, mix125µL (0.125 mL) of **E2-HRP concentrate** (number 7) to a total volume of 2.5 mL (0.125 mL + 2.375 mL) with **conjugate buffer** (number 8). This is sufficient for 100 EIA wells. Immediately after use, store the unused portion of the **Estradiol-HRP working reagent** at 2-8°C. Discard if not used within 3 weeks of mixing.

9. Wash solution (10X concentrated) EIA #1: 1 bottle, 50 mL. Prior to use dilute 1:10 with deionized water.
10. Color Development Reagent EIA #1: 1 amber plastic bottle, 15 mL of Tetramethyl-Benzidine (TMB) plus hydrogen peroxide. Light sensitive.
11. Stopping Solution EIA #1: 1 bottle 15 mL diluted acid solution.

V. Storage and Stability

- When stored at 2° – 8° C, unopened reagents will retain activity until the expiration date. Do not use the reagents beyond this date.
- Use only reagents supplied with this kit. Do not interchange reagents with different lot numbers.
- Opened reagents must be stored at 2° – 8° C.
- Microtiter wells must be stored at 2° – 8° C. Once the foil bag has been opened, care should be taken to reseal tightly.
- Opened kits retain activity for 3 weeks if stored as described above.
- Expiration dates and lot numbers are printed on the labels.

VI. Materials Needed but not Supplied

- Device to dispense very accurately 100 µL of saliva.
- Multichannel pipettors.
- Microplate or orbital shake
- Vortex mixer
- Microplate washer (not required, plates can be washed manually).

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- Microplate reader capable of reading 450 nm with 4 parameter data reduction or comparable software.
- Plate sealers.
- Collection device, VWR Sample Mailing Tubes, Cat #16465-260. (VWR International, 975 Overland Crt, San Dimas, CA 91773)

VII. Interferences

Commonly consumed products such as alcohol, caffeine, nicotine and food and gum extracts may interfere with Estradiol measurements. As a precautionary measure, follow the sample collection instructions as stated in section VIII. 1, under Collection.

VIII. Sample Collection and Processing

1. ***Collection:*** This sample collection and processing procedure must be followed.

- Avoid food consumption, drinking coffee or alcohol, smoking or chewing gum one (1) hour prior to sample collection.
- Rinse mouth thoroughly with water 15 minutes prior to collection.
- Collect whole saliva by unstimulated passive drool by allowing saliva to drip off the lower lip into a graduated plastic test tube (Pantex recommends VWR Sample Mailing Tubes Cat #16465-260) or by allowing saliva to accumulate in the floor of the mouth and spitting it into the recommended collection device.
- Time and date specimen. Refrigerate then freeze (-20°C or below) samples until day of assay. On day of assay, Thaw samples to facilitate precipitation of mucins. Centrifuge at 1500g for ten minutes. Bring samples to room temperature and assay.

2. ***Sample Stability:***

Storage	Room Temperature 20 – 30 °C	37 °C	2 – 8 °C	≤ -15 °C (7 freeze / thaw cycles)	≤ - 15 ° C (Long term)
Stability	Up to 7 days	Up to 7 days	Up to 7 days	Up to 7 days	Up to 9 months

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IX. Procedure Summary Flow Sheet

Calibrator Control Sample I.D.	Calibrator Control Sample (µL)	Estradiol HRP Working Reagent (µL)	Anti-Estradiol (µL)	Mix. Incubate for 120 min. at Room Temperature, shaking.	Diluted 10X Wash Solution. (µL)	Wash 3X	Color Development Reagent (µL)	Mix. Incubate 30 min. at room temperature	Stopping Solution (µL)	Mix. Read at 450 nm
0	100	25	25		300		125		125	
1	100	25	25		300		125		125	
2	100	25	25		300		125		125	
4	100	25	25		300		125		125	
8	100	25	25		300		125		125	
16	100	25	25		300		125		125	
32	100	25	25		300		125		125	
Control #1	100	25	25		300		125		125	
Control #2	100	25	25		300		125		125	
Sample #1	100	25	25		300		125		125	
Sample #2	100	25	25		300		125		125	
Sample #3	100	25	25		300		125		125	

X. Assay Procedure

1. It is recommended that the **calibrators, controls** and **samples** should be tested in duplicate and the mean value should be used to report results.

2. To the GARGG microplate dispense **100 µL** of the previously prepared working **Estradiol Calibrators** (0, 1, 2, 4, 8, 16 and 32 pg/mL), **controls**, and **saliva samples**. Mix the microplate by shaking gently (manual) for a few seconds.

3. Add **25 µL** of **Estradiol HRP Working Reagent.**

4. Add **25 µL** of **Anti-Estradiol EIA Antibody.**

5. Cover the microplate with plastic sealer. Incubate by shaking on a microplate orbital shaker set at 500 – 900 rpm for **2 hours** at room temperature.

6. After incubation decant content of the wells. Wash 3 times with 300 µL of diluted Wash Solution (**10 mL** of 10XWash solution EIA #1 diluted with **90 mL** of D.I. water) after the 3rd wash invert GARGG microplate on absorbent paper and tap dry.

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7. Dispense 125 µL of Color Development reagent EIA #1 into each well. Shake briefly (manual). Cover microplate with plastic sealer. Incubate for **30 minutes** at room temperature.

8. Dispense 125µL of Stopping Solution EIA #1 into each microtiter well of the GARGG plate. Shake briefly (manual). Color changes from blue to yellow.

9. Read at 450 nm on a microplate reader within **10 minutes**.

   Note: If samples exceed the highest calibrator, dilute with zero calibrator and make appropriate concentration correction.

XI. Typical results.

Typical Calibration Curve for a 100 µL Sample Size (Actual assay)			
Calibrators (pg/mL)	**Mean Absorbance (450 nm)**	**% B/Bo**	**Value (pg/mL)**
0	2.16	100.0	0
1	1.82	84.3	1
2	1.65	76.4	2
4	1.33	61.6	4
8	0.86	39.8	8
16	0.50	23.1	16
32	0.32	14.8	32
Control #1	1.53	70.8	2.5
Control #2	0.73	33.8	10.7
Sample #1	1.65	76.4	1.9
Sample #2	0.33	15.3	28.2
Sample #3	0.97	44.9	6.9

XII. Calculation

1. Compute the average optical density (OD) for the zero (Bo) calibrator.

2. Calculate the percent bound (B/Bo) for each calibrator, control and unknown by dividing the average OD (B) by the average OD for the zero (Bo) x 100.

3. Plot percent bound (B/Bo) versus the calibrator concentrations and draw the best fit for the curve.

4. Plot percent bound (B/Bo) of the controls and unknowns to determine saliva estradiol concentrations.

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5. Alternately determine the concentrations of the controls and unknowns by interpolation using software capable of logistics using a 4-parameter sigmoid minus curve fit.

Analytical measuring range (AMR)	1 pg/mL – 32 pg/mL

Samples with estradiol values greater than 32 pg/mL should be diluted 1:10 with zero (0) calibrator and re-run for accuracy. Obtain the final estradiol concentration by multiplying the diluted sample by the dilution factor.

XIII. Quality Control

The expected values for the controls are stated on the label of each control which are included in the kit. The results can only be accepted if the expected values are met.

XIV. Expected Values

The following AM values were obtained from apparently healthy subjects with the Pantex Direct Salivary 17β-Estradiol EIA Kit, Cat #674

Estradiol (pg/mL)		**Median**	**Range**
Females Premenopausal (not using contraceptives) n=90, Age 21- 50 yrs	Follicular Phase	3.16	2.13 – 5.6
	Luteal Phase	3.15	1.42 – 14.43
Females Postmenopausal n=51, Age 51 – 75 yrs		2.53	0.80 – 4.07
Males n=43, Age 20 – 70 yrs		2.68	1.07 – 5.00

It is recommended that each laboratory establish its own normal ranges.

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XV. Performance Characteristics

A. Specificity of Antiserum:

C-18 Steroids:	Spiked Concentration	% Cross reactivity
Estradiol-17ß	10,000 pg/mL	100.00
Estradiol-17α	10,000 pg/mL	0.0112
Estriol	10,000 pg/mL	0.0615
Estrone	10,000 pg/mL	0.9225
Estrone-3-SO4	10,000 pg/mL	0.3398
Estriol-3-SO4	10,000 pg/mL	0.0066
C-19 Steroids:	**Spiked Concentration**	**% Cross reactivity**
Androstenedione	10,000 pg/mL	0.0008
Testosterone	10,000 pg/mL	0.0082
5α-Dihydrotestosterone	10,000 pg/mL	0.0050
Dehydroepiandrosterone SO4	10,000 pg/mL	0.0030
Androstanedione	10,000 pg/mL	0.0148
C-21 Steroids:	**Spiked Concentration**	**% Cross reactivity**
Progesterone	10,000 pg/mL	<0.001
17-OH Progesterone	10,000 pg/mL	0.0048
Pregnenolone	10,000 pg/mL	0.0044
17-OH-Pregnenolone	10,000 pg/mL	0.0096
Desoxycorticosterone	10,000 pg/mL	0.0063
11-Desoxycortisol	10,000 pg/mL	0.0085
Corticosterone	10,000 pg/mL	0.0171
Aldosterone	10,000 pg/mL	0.0072
Cortisol	10,000 pg/mL	0.0104

B. Detection Limits:

The LOB (limit of the blank) and the LOD (limit of detection) were determined by generating one hundred twenty (120) measurements each of “estradiol free salvia” and low level (<1 pg/mL) estradiol samples (Reference, CLSI EP 17-A, protocols for Determination of Limits of Detection and Limits of Quantitation).

Limit of the Blank (LoB) pg/mL	Limit of Detection (LoD) pg/mL
0.3680	0.5239

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C. Precision and Reproducibility:

Intra-assay

The intra-assay precision was determined from the mean of 20 replicates of low, medium and high samples.

Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	% CV
Low	20	3.29	0.352	10.7
Medium	20	27.24	0.999	3.7
High	20	52.56	3.103	5.9

Inter-assay

The inter-assay precision was determined from the mean of the average duplicates of 12 separate assays with low, medium and high samples.

Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	% CV
Low	12	3.01	0.266	8.8
Medium	12	26.13	0.939	3.6
High	12	49.19	4.552	9.3

Repeatability

This study was conducted during 5 days of a familiarization period and 20 days of testing. Two assays were performed daily. Three (3) different reagent lots and three (3) saliva pools were used for the study (low, medium and high). The pools were aliquoted and frozen until day of assay.

Precision Low Concentration Pool

	Standard Deviation, (SD)	% Coefficient of Variation, (CV)
Within Run	0.047	1.7
Between Run	0.052	1.9
Repeatability	0.105	3.9
Total Device Precision	0.126	4.6

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Precision Medium Concentration Pool

	Standard Deviation, (SD)	% Coefficient of Variation, (CV)
Within Run	0.323	1.2
Between Run	0.096	0.4
Repeatability	0.552	2.1
Total Device Precision	0.647	2.5

Precision High Concentration Pool

	Standard Deviation, (SD)	% Coefficient of Variation, (CV)
Within Run	0.321	0.7
Between Run	0.964	2.1
Repeatability	0.859	1.8
Total Device Precision	1.331	2.8

Inter-lot Variation

It was determined by testing 10 pools of saliva samples and 2 controls with 3 different lots of reagents.

Samples ID	Lot # 001 (pg/mL)	Lot # 002 (pg/mL)	Lot # 003 (pg/mL)	Inter-lot Mean (pg/mL)	Inter-lot SD (pg/mL)	Inter-lot CV (%)
40	9.89	9.28	9.95	9.71	0.371	3.8
41	3.06	3.34	3.20	3.20	0.140	4.4
42	2.27	2.37	2.35	2.33	0.053	2.3
43	2.18	2.61	2.54	2.44	0.231	9.4
44	4.78	4.81	4.88	4.82	0.051	1.1
45	6.80	6.72	6.80	6.77	0.046	0.7
46	4.31	4.51	4.52	4.45	0.118	2.7
LP	2.56	2.65	2.56	2.59	0.052	2.0
MP	28.36	29.25	27.89	28.50	0.691	2.4
HP	47.82	49.44	47.61	48.29	1.001	2.1
C1	4.00	3.71	3.75	3.82	0.157	4.1
C2	10.67	10.53	10.58	10.59	0.071	0.7

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D. Linearity Study:

Linearity Study Data
S=10 samples (dilutions)
Concentration = (C1*V1+C10*V10)/(V1+V10)

	C1 (pg/mL)	V1 (mL)	C10 (pg/mL)	V10 (mL)	Calculated Concentration (pg/mL)	Obtained Concentration (pg/mL)	Recovery (%)
1				*	0.40	0.42	105
2	0.42	0.889	81.57	0.111	9.43	8.44	90
3	0.42	0.778	81.57	0.222	18.44	16.61	90
4	0.42	0.667	81.57	0.333	27.44	24.88	91
5	0.42	0.556	81.57	0.444	36.45	36.32	100
6	0.42	0.444	81.57	0.556	45.54	45.50	100
7	0.42	0.333	81.75	0.667	54.55	56.08	103
8	0.42	0.222	81.75	0.778	63.55	65.69	103
9	0.42	0.111	81.57	0.889	72.56	76.66	106
10				*	80.00	81.57	102
* Targets of low and high sample concentrations.							

E. Recovery:

Seven (7) saliva samples containing different levels of endogenous estradiol were spiked with known quantities of estradiol, assayed and the percent recovery determined.

Sample	Endogenous (pg/mL)	Estradiol Added (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	1.00	64.0	65.00	60.37	92.9
2	3.20	32.0	35.20	34.53	98.1
3	4.42	16.0	20.42	19.55	95.7
4	4.24	8.0	12.24	11.53	94.2
5	24.61	4.0	28.61	26.82	93.7
6	40.90	2.0	42.90	42.02	97.9
7	4.86	1.0	5.86	5.30	90.4

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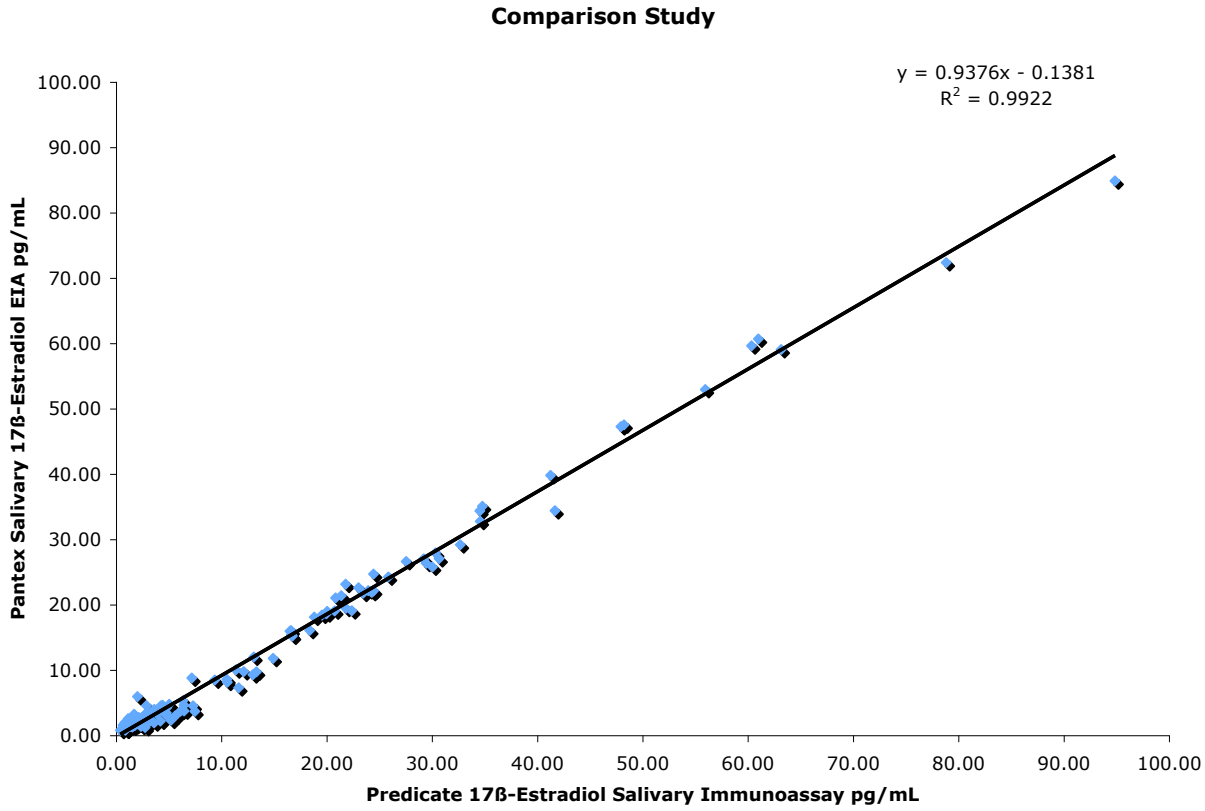
F. Comparison Study:

One hundred twenty-five (125) saliva samples spanning the analytical measuring range (AMR) and beyond were analyzed by comparing the Pantex Direct Salivary 17β-Estradiol EIA Kit, Cat #674 with a commercially available predicate Estradiol salivary Immunoassay. See correlation results below.



XVI. Limitations

- The reagents are optimized to measure 17β-estradiol directly in human saliva.
- Samples containing sodium azide are not suitable for this assay.
- Avoid blood contamination of samples. Do not collect samples when oral diseases, inflammation or lesions exist.
- Improper handling of samples or modification of this assay might influence the results.

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XVII. Precautions

- Only physician, clinical labs, research labs and hospital labs may acquire, possess and use the kit.
- Compare contents and packing list, if there is breakage or shortage, notify Pantex immediately.
- Do not pipet reagents by mouth.
- Do not smoke, eat or drink while performing assay.
- Wear disposable rubber gloves.
- Treat all saliva samples as potentially infectious.
- Do not mix reagent lot numbers or alter in any way the reagents in this kit. If this is done, Pantex will not be responsible for the performance of the assay.
- Avoid contact with Color Development Reagent (TMB). It contains solvents that can irritate skin and mucus membranes. If contact is made, wash thoroughly with water.
- Avoid contact with stopping solution. It contains acid. If contact is made, rinse thoroughly with water.

XVIII. References

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