STELLUX™

C-peptide Chemiluminescence ELISA

For the quantitative determination of Human C-peptide in Human Serum, Heparin Plasma, EDTA plasma, and Tissue Culture Supernatants.

For *In Vitro* Diagnostic use within the United States of America.
This product is for Research Use Only outside of the United States of America.

Catalog Number:	80-CPTHU-CH01, CH05
Size:	96 wells, 5 x 96 wells
Version:	January 30, 2018

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INTENDED USE

The ALPCO STELLUX™ C-peptide Chemiluminescence ELISA is designed for the quantitative determination of C-peptide in human serum, heparin plasma, EDTA plasma, and tissue culture supernatants. For In Vitro Diagnostic Use.

PRINCIPLE OF THE ASSAY

The ALPCO STELLUX™ C-peptide Chemiluminescence ELISA is a sandwich type immunoassay. The 96-well microplate is coated with a monoclonal antibody specific for C-peptide. The standards, controls, and samples are added to the microplate wells in addition to Assay Buffer. The microplate is then incubated at room temperature on a microplate shaker at 700-900 rpm. After the first incubation is complete, the wells are washed with Wash Buffer and blotted dry. The detector antibody is then added, and the microplate is incubated a second time on a microplate shaker at 700-900 rpm, washed, and blotted dry. Streptavidin-HRP is added to the wells for a third incubation at room temperature on a microplate shaker at 700-900 rpm, washed, and blotted dry. Chemiluminescent Substrate is added, and the microplate is read by a luminescence plate reader at one minute. The intensity of the light generated is directly proportional to the amount of C-peptide in the sample.

MATERIALS SUPPLIED

80-CPTHU-CH01		
Component	**Quantity**	**Preparation**
C-peptide Microplate (96 wells)	12 x 8 strips	Ready to use
Standards (A-H)* (4.5, 9, 18, 36, 144, 720, 4320, 12960 pg/mL)	1 vial each	Lyophilized*
Control Levels 1, 2 and 3*	1 vial each	Lyophilized*
Assay Buffer	14 mL	Ready to use
Detector Antibody	150 µL	101X
Detector Antibody Buffer	12 mL	Ready to use
Streptavidin-HRP	150 µL	101x
Streptavidin-HRP Buffer	12 mL	Ready to use
Wash Buffer Concentrate	100 mL	21X
Substrate A	6 mL	Ready to use
Substrate B	6 mL	Ready to use
Plate Sealers	3	Ready to use

*Please refer to the Certificate of Analysis enclosed with each kit for lot specific standard concentrations, control ranges, and reconstitution volumes.

80-CPTHU-CH05		
Component	**Quantity**	**Preparation**
C-peptide Microplate (96 wells)	5 x (12 x 8 strips)	Ready to use
Standards (A-H)* (4.5, 9, 18, 36, 144, 720, 4320, 12960 pg/mL)	1 vial each	Lyophilized*
Control Levels 1, 2 and 3*	1 vial each	Lyophilized*
Assay Buffer	70 mL	Ready to use
Detector Antibody	630 µL	101X
Detector Antibody Buffer	60 mL	Ready to use
Streptavidin-HRP	630 µL	101x
Streptavidin-HRP Buffer	60 mL	Ready to use
Wash Buffer Concentrate	2 x 200 mL	21X
Substrate A	30 mL	Ready to use
Substrate B	30 mL	Ready to use
Plate Sealers	15	Ready to use

*Please refer to the Certificate of Analysis enclosed with each kit for lot specific standard concentrations, control ranges, and reconstitution volumes.

MATERIALS REQUIRED

- Precision pipettes for dispensing 10-100 µL (with disposable tips)
- Repeating or multi-channel pipette for dispensing up to 100 µL
- Volumetric containers and pipettes for reagent preparation
- Distilled or deionized water for reagent preparation
- Microplate washer or wash bottle
- Microplate shaker capable of 700-900 rpm
- Microplate reader with luminometer
- Vortex for Sample Preparation

PRECAUTIONS

1. The human blood products incorporated into this kit have been tested for the presence of HIV (Human Immunodeficiency virus), HBV (Hepatitis B virus), and HCV (Hepatitis C virus). Test methods for these viruses do not guarantee the absence of a virus; therefore, all reagents should be treated as potentially infectious. Handling and disposal should be in accordance with all appropriate national and local regulations for the handling of potentially bio-hazardous materials.
2. All materials derived from animal sources are BSE negative. However, all materials should be treated as potentially infectious.
3. Avoid direct contact with skin.
4. This product is not for internal use.
5. Avoid eating, drinking, or smoking when using this product.
6. Do not pipette any reagents by mouth.
7. Reagents from this kit are lot-specific and must not be substituted.
8. Do not use reagents beyond the expiration date.
9. Variations to the test procedure are not recommended and may influence the test results.
10. ***An appendix has been included with examples of instrument settings for reading a chemiluminescent output*.** Each lab should optimize their instrument settings according to the manufacturer's instructions. Please contact the technical services department of the manufacturer of the microplate reader for optimal instrument settings.
11. The assay requires that all wash steps consist of 6 washes, with a 1 minute soak in between each wash. ***The soaking steps should not be omitted.***

STORAGE CONDITIONS

The kit should be stored at 2-8°C. The kit is stable until the expiration date on the box label. If multiple assays are to be performed, reconstituted standards and controls should be stored in aliquots at -20°C. Avoid repeated freeze/thaw cycles.

SAMPLE HANDLING

Serum and plasma samples are appropriate for use in this assay. No dilution or treatment of the sample is required. However, if a sample has a greater concentration of C-peptide than the highest standard, the sample should be diluted in Assay Buffer and the analysis should be repeated.

Tissue culture supernatants, serum-free tissue culture media, and tissue culture media containing serum (complete media) are also appropriate for use in this assay. Supernatants from cultured islet cells should be diluted 1:20 in the assay buffer.

It is recommended to ***1)*** thoroughly vortex each sample before use, ***2)*** spin the samples at 13,000rpm for 10 minutes before use, and ***3)*** perform pipetting actions without pausing.
Samples can be stored at 2-8°C for 24 hours prior to analysis in this assay. For longer periods, storage at ≤ -80°C is recommended. Avoid repeated freeze/thaw cycles of samples.

REAGENT PREPARATION

All reagents must be equilibrated to room temperature prior to preparation and subsequent use in the assay. Prepare reagents according to the number of plates or strips to be used. Store the remaining concentrates at 2-8ºC.

Standards (A-H) are provided in lyophilized form. Please refer to the Certificate of Analysis provided with each kit for the appropriate volume of deionized water for reconstitution. If multiple assays are to be performed, reconstituted standards should be stored in aliquots at ≤ -20°C. Avoid repeated freeze/thaw cycles.

Controls (Levels 1, 2 and 3) are provided in a lyophilized form. Please refer to the Certificate of Analysis provided with each kit for the appropriate volume of deionized water for reconstitution. Close the vial with the rubber stopper and cap, gently swirl the vial, and allow it to stand at room temperature for 30 minutes prior to use. The contents of the vial should be in solution with no visible particulates. The reconstituted controls can be stored at ≤ -20°C in aliquots for up to 3 months. The controls should not be repeatedly frozen and thawed.

Detector Antibody Stock (101X) is to be diluted with 100 parts Detector Antibody Buffer. Working Strength Detector Antibody is stable for 8 hours at 2-8°C.

Number of plates	Amount of Concentrate	Amount of Detector Antibody Buffer
0.5 (6 strips)	60 µL	6 mL
1 (12 strips)	120 µL	12 mL
2	240 µL	24 mL
5	600 µL	60 mL
10	1200 µL	120 mL

Streptavidin-HRP Concentrate (101X) is to be diluted with Streptavidin-HRP Buffer. Working Strength Streptavidin-HRP is stable for 8 hours at 2-8°C.

Number of plates	Amount of Concentrate	Amount of SA-HRP Buffer
0.5 (6 strips)	60 µL	6 mL
1 (12 strips)	120 µL	12 mL
2	240 µL	24 mL
5	600 µL	60 mL
10	1200 µL	120 mL

Wash Buffer Concentrate (21X) is to be diluted with 20 parts distilled water. Working Strength Wash Buffer is stable for 4 weeks at room temperature.
NOTE: All labs should account for plate washer void volume to prime the machine.
The values below ***do not*** account for priming automated plate washers.

Number of plates	Amount of Concentrate (6 x 350 µL wash)	Amount of dH_2O (6 x 350 µL wash)
0.5 (6 strips)	15 mL	300 mL
1 (12 strips)	30 mL	600 mL
2	50 mL	1,000 mL
5	130 mL	2,600 mL
10	250 mL	5,000 mL

Substrates A & B are provided individually and should be combined in equal parts to create the Working Chemiluminescence Substrate immediately before use.

Number of plates	Amount of Substrate A	Amount of Substrate B
0.5 (6 strips)	3 mL	3 mL
1 (12 strips)	6 mL	6 mL
2	12 mL	12 mL
5	30 mL	30 mL
10	60 mL	60 mL

QUALITY CONTROL

It is recommended that the Controls provided with the STELLUX™ C-peptide Chemiluminescence ELISA be included in every assay. The concentration ranges of the controls are provided on the Certificate of Analysis provided with each kit; however, it is recommended that each laboratory establishes its own acceptable ranges.

ASSAY PROCEDURE

All reagents and microplate strips should be equilibrated to room temperature prior to use. Gently mix all reagents before use. A standard curve must be performed with each assay run and with each microplate if more than one is used at a time. All standards, controls, and samples should be run in duplicate. A suggested plate layout is provided.

1. The microplate should be equilibrated to room temperature prior to opening the foil pouch. Designate enough microplate strips for duplicate determinations of the standards, controls, and samples. The remaining microplate strips should be stored at 2-8°C tightly sealed in the foil pouch containing the desiccant.
2. **Pipette 50 µL** of each standard, control, and sample into their respective wells. The Assay Buffer is used for the Zero Standard (blank) in the assay. See *Reagent Preparation* and Certificate of Analysis for standard and control reconstitution instructions. Note: for tissue culture supernatants, see *Sample Handling.*
3. **Pipette 50 µL** of Assay Buffer into each well.
4. Cover microplate with a plate sealer and **incubate for 1 hour** at room temperature, shaking at 700-900 rpm on a microplate shaker.
5. Decant the contents of the wells and **wash the microplate 6 times, soaking for 1 minute after each wash** with 350 µL of Working Strength Wash Buffer per well (see *Reagent Preparation*) using a microplate washer. ***Do not omit the soaking steps.***
    a. Alternatively, fill the wells with Working Strength Wash Buffer using a wash bottle equipped with a wash nozzle (manual washer). (It is not recommended to use a multichannel pipette. Wash buffer must be dispensed with adequate and equal force in order to properly wash the wells.) Soak the wells for 1 minute. Invert the microplate to discard the liquid and firmly tap the inverted microplate on absorbent paper towels. Repeat the wash and soak procedure 5 more times, for a total of 6 washes.

    After the final wash, (automated or manual), remove any residual Wash Buffer and bubbles from the wells by inverting and firmly tapping the microplate on absorbent paper towels.

6. **Pipette 100 µL** of Working Strength Detector Antibody Solution (see *Reagent Preparation*) into each well.
7. Cover microplate with a plate sealer and **incubate for 1 hour** at room temperature, shaking at 700-900 rpm on a microplate shaker.
8. Decant the contents of the wells and **wash the microplate 6 times** with 350 µL of Working Strength Wash Buffer per well. See Step 5.
9. **Pipette 100 µL** of Working Strength Streptavidin-HRP solution (see *Reagent Preparation*) into each well.
10. Cover microplate with a plate sealer and **incubate for 30 minutes** at room temperature, shaking at 700-900 rpm on a microplate shaker.
11. Decant the contents of the wells and **wash the microplate 6 times** with 350 µL of Working Strength Wash Buffer per well. See Step 5.
12. **Pipette 100 µL** of Working Chemiluminescent Substrate (see *Reagent Preparation*) into each well.
13. Place the microplate in a microplate reader capable of reading the luminosity of the wells. The microplate should be analyzed at 1 minute after the addition of the Chemiluminescent Substrate, and no more than 10 minutes after substrate addition. Read the plate using a 1 second integration time.

CALCULATION OF RESULTS

Construct a standard curve from the standards. It is recommended to use a software program to calculate the standard curve and to determine the concentration of the samples. A 5-parameter curve fit with $1/y^2$ weighting is recommended for data analysis.

The STELLUX™ C-peptide Chemiluminescence ELISA is a ligand binding assay, with responses exhibiting a sigmoidal relationship to the analyte concentration. Currently accepted reference models for such curves use a 4- or 5-parameter logistic (pl) fit, as these models optimize the accuracy and precision across a greater range.

A 5-pl curve fit with $1/y^2$ weighting is used to maximize the accuracy and precision of samples with low concentrations. However, the accuracy and precision of all models are limited at the lowest and highest ends of the detectable range due to the influence of individual laboratory conditions. As a result, caution should always be used when interpreting results where the analyte response becomes non-linear.[1]

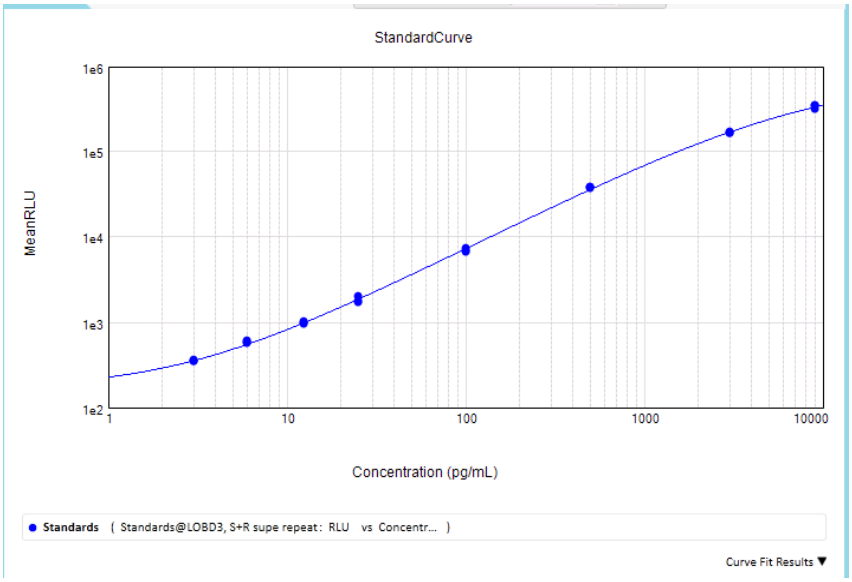
Extrapolating sample concentration values outside the range of the standard concentration values is not recommended.

For any sample that is diluted, multiply the determined concentration by the dilution factor.

TYPICAL STANDARD CURVE

The following results are provided for demonstration purposes only and cannot be used in place of data obtained with the assay. A standard curve with controls must be run with each assay. A 5-parameter curve fit with $1/y^2$ weighting is used for data analysis.

Standard	Conc. pg/mL	Conc. pM	RLU
A	12,960	4,320	336,053
B	4,320	1,440	166,427
C	720	240	38,054
D	144	48	6,933
E	36	12	1,817
F	18	6	979
G	9	3	585
H	4.5	1.5	347
Zero	0	0	168



EXPECTED VALUES

The STELLUX™ C-peptide Chemiluminescence ELISA is calibrated to the WHO C-peptide 1st IRP 84/510. It is recommended that each laboratory establish its own normal range for its individual patient population.

Conversion for Human C-peptide from pg/mL to pM: 3 pg/mL = 1 pM

ASSAY DEVELOPMENT TIME

It is recommended to read the plate no later than 10 minutes after the addition of substrate to the wells. The control concentration values have been shown to vary less than ±10% from their value at a 1 minute read for up to 10 minutes post substrate addition.

	Calculated Concentration (pg/mL)		
Control	**1 minute**	**5 minutes**	**10 minutes**
Level 1	36.5	37.8	39.6
Level 2	275	272	279
Level 3	3025	3049	3062

PERFORMANCE CHARACTERISTICS

(Refer to the lot specific Certificate of Analysis for the most relevant performance data.)

Sensitivity

The Limit of Blank (LoB), Limit of Detection (LoD), and the Limit of Quantitation (LoQ), were determined in accordance with the CLSI guideline EP17-A.

Limit of Blank (LoB) is defined as the concentration obtained by plotting the mean RLU + 1.645*SD of 60 Zero standard replicates over three days against the standard curve.

Limit of Detection (LoD) is defined as the concentration obtained by plotting the calculated LOB RLU+ 1.645*SD of the same 60 replicates over three days.

The LoB and LoD of the assay are less than or equal to 4.32 pg/mL.

The limit of quantitation (LoQ) is defined as the lowest concentration which has a calculated concentration CV ≤20%, and an accuracy of 80-120%. The LoQ of the assay is less than or equal to 18 pg/mL.

Precision: Within run (intra-assay) variation

The within run precision is expressed as the percentage coefficient of variation (CV (%)). This was determined based on the mean and standard deviation of 16 replicates of a sample run in a single assay. The table below shows the results of 3 samples that span the range of the assay.

	Sample 1	Sample 2	Sample 3
Mean	7692 pg/mL	561 pg/mL	39.7 pg/mL
CV (%)	12.2	8.6	8.5
n	16	16	16

Precision: Between run (inter-assay) variation
The between run precision is expressed as the percentage coefficient of variation (CV %). This was determined based on the mean and standard deviation across 2 assays of 36 measurements of a single sample. The table below shows the results of 3 samples that span the range of the assay.

	Sample 1	Sample 2	Sample 3
Mean	7841 pg/mL	584 pg/mL	43.1 pg/mL
CV (%)	13.3	9.2	11.7
n	32	32	32

Linearity
The linearity of the assay was determined by preparing dilutions of samples with high C-peptide concentrations 1:2 to 1:128 with the Zero Standard. The expected values were compared to the observed values to determine a percent recovery. The mean recovery for serum samples is 102.1% (range 95.8-105.8%). The mean recovery for EDTA plasma samples is 109.3% (range 94.8-115.2%). The mean recovery for heparin plasma samples is 106.9% (range 100.0-114.7%). The mean recovery of islet cell culture supernatants is 108.1% (range 102.5-113.5%).
NOTE: Supernatants from cultured islet cells were diluted 1:20 in the assay buffer prior to performing linearity dilutions.

Spike and Recovery
The spike and recovery of the assay was determined by adding various known amounts of C-peptide to samples. Serum, heparin plasma, EDTA plasma, and islet cell culture supernatants were evaluated. The expected values were compared to the observed values to determine a percent recovery. The mean and range of recovery for the samples are listed below:

		Mean recovery (%)	Min (%)	Max (%)
Serum	low	104.4	85.2	123.0
	mid	105.3	80.8	116.5
	high	100.1	78.3	121.0
Heparin plasma	low	97.2	91.9	103.1
	mid	97.4	93.0	101.8
	high	99.4	85.1	108.3
EDTA Plasma	low	93.6	85.0	102.4
	mid	90.8	77.7	100.6
	high	108.2	96.3	111.7
Cell culture supernatant	low	102.1	99.2	103.1
	mid	87.7	83.5	91.9
	high	97.9	95.2	101.4

Specificity
The table below indicates the analyte and the percent cross-reactivity observed in the assay.

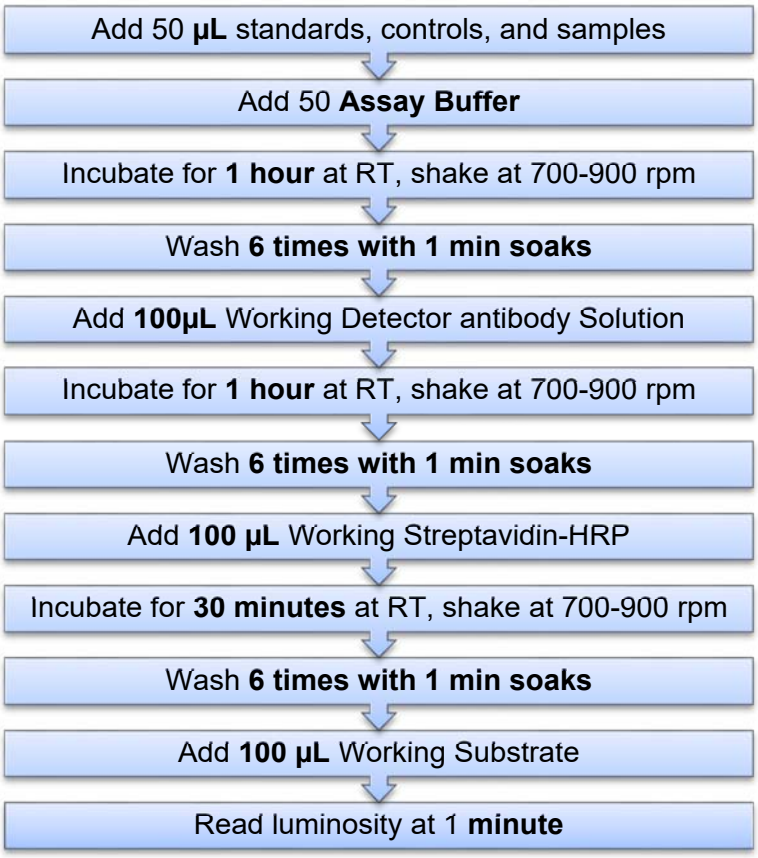
Analyte	% Cross-reactivity
Human Insulin	Not Detected
Human Proinsulin (intact)	<0.01
Human Proinsulin Des(32,33)	0.3
Human Proinsulin Des(64,65)	33.2
Humalog	Not Detected
Novolog	Not Detected
Humulin R	Not Detected
Porcine Insulin	Not Detected
Porcine C-peptide	Not Detected
Rat C-peptide I	Not Detected
Rat C-peptide II	Not Detected
Mouse C-peptide I	Not Detected
Mouse C-peptide II	Not Detected
Rat Proinsulin I	Not Detected
Rat Proinsulin II	Not Detected
Mouse Proinsulin I	Not Detected
Mouse Proinsulin II	Not Detected

Hook Effect
No high dose hook effect was observed with C-peptide concentrations up to 300,000 pg/mL.

REFERENCES

1. Finlay JWA, Dillard RF. Appropriate Calibration Curve Fitting in Ligand Binding Assays. *AAPS Journal*. 2007; 9(2): E260-E267.

SHORT ASSAY PROTOCOL



Total Incubation Time = 2 hour 31 minutes

SUGGESTED PLATE LAYOUT

Below is a suggested plate layout for running standards, controls, and up to 36 samples in duplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std A	Std A	Zero	Zero	5	5	13	13	21	21	29	29
B	Std B	Std B	Ctrl 1	Ctrl 1	6	6	14	14	22	22	30	30
C	Std C	Std C	Ctrl 2	Ctrl 2	7	7	15	15	23	23	31	31
D	Std D	Std D	Ctrl 3	Ctrl 3	8	8	16	16	24	24	32	32
E	Std E	Std E	1	1	9	9	17	17	25	25	33	33
F	Std F	Std F	2	2	10	10	18	18	26	26	34	34
G	Std G	Std G	3	3	11	11	19	19	27	27	35	35
H	Std H	Std H	4	4	12	12	20	20	28	28	36	36

Std= Standard
Ctrl = Control
Zero = Assay Buffer
Numbered wells = Samples

APPENDIX

Instrument settings: Please contact the microplate reader manufacturer's technical services department for additional assistance. These instrument settings are to be used as a guideline. It is optional to shake the plates before reading for ≤ 3 seconds.

Molecular Devices Spectramax L
Read Mode: Luminescence
Integration Time: 1 second (1000 ms)
Sensitivity:
PMT: MaxRange
Target Calibration Wavelength: 470 nm
Automix: Classic: 30 mm/s
Automix before read: Off
Plate Type: 96 well standard
Injection and Delay: Off
Injection wells: None
Dark Adapt: Off
AutoRead: Off

Molecular Devices Spectramax M5
Read Mode: Luminescence (LUM)
Read Type: Endpoint
Wavelength: All
Plate Type: 96 well standard opaque
Read Area: Variable based on experiment
PMT and Optics: Integration Time 1000 ms
Shake: Off
More Settings: Calibrate (on); Carriage Speed (Normal); Read Order (Column); Setting Time (off)

Biotek Synergy2
Detection Method: Luminescence
Read Type: Endpoint
Integration: 0:01.00 (MM:SS.ss) (1 second)
Emission: Hole
Optics Position: Top
Sensitivity: 150
Top Probe Vertical Offset: 1.00mm

Tecan Infinite 200
Plate: Corning 96 Flat Bottom Black Polystyrol
Mode: Luminescence
Attenuation: NONE
Integration Time: 1000 ms
Settle Time: 0 ms