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ab205715 – STAT5 A/B (pY694/699) + Total STAT5 A/B SimpleStep ELISA® Kit

For the semi-quantitative measurement of STAT5 A/B (pY694/699) and Total STAT5 A/B in Human cell lysates.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Abcam's STAT5 A/B (pY694/699) and STAT5 A/B (Total) in vitro SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit (ab205715) is designed for the semi-quantitative measurement of STAT5 A/B (pY694/699) and Total STAT5 A/B protein in Human cells.

The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB substrate is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at 600 nm.

STAT5 A and STAT5 B (collectively known as STAT5 A/B) are two very closely related proteins. Although encoded by different genes, STAT5 A and STAT5 B share 96% identity at the protein level. Similarly to other STAT proteins, STAT5 A and STAT5 B are activated by tyrosine phosphorylation, usually by JAK proteins, at Tyr694 and Tyr699, respectively.

STAT5 A and STAT5 B show differential, cell-specific regulation, with STAT5 A expression predominant in mammary tissue, while STAT5 B expression is more abundant in muscle and liver tissues. STAT5 A/B plays an integral role in immune cell development and regulation, and is an important mediator of IL-2 and IL-15 signaling in regulatory T cells.

2. Protocol Summary

Prepare all reagents, samples, and standards as instructed

Add standard or sample to appropriate wells.

Add Antibody Cocktail to all wells. Incubate at room temperature.

Aspirate and wash each well.

Add TMB Substrate to each well and incubate.

Add Stop Solution at a defined endpoint.

Alternatively, record color development kinetically after TMB substrate addition.

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at +4°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage Condition
STAT5 A/B (pY694/699) Capture Antibody	1.5 mL	+2-8°C
STAT5 A/B (pY694/699) Detector Antibody	1.5 mL	+2-8°C
STAT5 A/B (Total) Capture Antibody	1.5 mL	+2-8°C
STAT5 A/B (Total) Detector Antibody	1.5 mL	+2-8°C
Lyophilized STAT5 A/B Control Lysate	1 Vial	+2-8°C
10X Wash Buffer PT	15 mL	+2-8°C
5X Cell Extraction Buffer PTR	10 mL	+2-8°C
50X Cell Extraction Enhancer Solution	1 mL	+2-8°C
TMB Substrate	12 mL	+2-8°C

Stop Solution	12 mL	+2-8°C
SimpleStep® Pre-Coated 96 Well Microplate (12 x 8 well strips)	96 Wells	+2-8°C
Plate seal	1	+2-8°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- An alternative fluorescent substrate, ADHP, can be used with this assay. Please see ab205810 ADHP HRP Substrate Kit for more information. A microplate reader capable of measuring fluorescence is required if using this product.
- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- PBS (1.4 mM KH2PO4, 8 mM Na2HPO4, 140 mM NaCl, 2.7 mM KCl, pH 7.4).
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps

8. Technical Hints

- This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.
- The provided 50X Cell Extraction Enhancer Solution may precipitate when stored at + 4°C. To dissolve, warm briefly at + 37°C and mix gently. The 50X Cell Extraction Enhancer Solution can be stored at room temperature to avoid precipitation.
- The provided 5X Cell Extraction Buffer contains phosphatase inhibitors. Protease inhibitors can be added if required.
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. **The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.**
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the formulations provided.

9.1 1X Cell Extraction Buffer PTR

Prepare 1X Cell Extraction Buffer PTR by diluting 5X Cell Extraction Buffer PTR and 50X Cell Extraction Enhancer Solution to 1X with deionized water. To make 10 mL 1X Cell Extraction Buffer PTR combine 7.8 mL deionized water, 2 mL 5X Cell Extraction Buffer PTR and 200 µL 50X Cell Extraction Enhancer Solution Mix thoroughly and gently. If required protease inhibitors can be added.

Alternative – Enhancer may be added to Cell Extraction Buffer after extraction of cells or tissue. Refer to note in Section 19.

9.2 5X Cell Extraction/Enhancer Buffer PTR

Prepare 5X Cell Extraction/Enhancer Buffer PTR by adding 1/10th volume of 50X Cell Extraction Enhancer Solution. To prepare 1 mL, add 100 µL of 50X Cell Extraction Enhancer Solution to 900 µL of 5X Cell Extraction Buffer PTR. This concentrated mix is used for lysing cells directly in cell culture medium.

9.3 1X Wash Buffer

Prepare 1X Wash Buffer PT by diluting 10X Wash Buffer PT with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL 10X Wash Buffer PT with 45 mL deionized water. Mix thoroughly and gently.

9.4 Antibody Cocktail

Prepare Antibody Cocktail by combining an appropriate volume of the capture and detector antibodies immediately prior to assay. To make 3 mL of the Antibody Cocktail combine 1.5 mL Capture Antibody with 1.5 mL Detector Antibody. Mix thoroughly and gently.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).

10.1 Reconstitute the Lyophilized STAT5 A/B Control Lysate* by adding 250 µL water by pipette. Mix thoroughly and gently. Hold at room temperature for 1 minute. This is the 100% Stock Lysate Solution (see table below). Use undiluted as **Control lysate #1.** Remaining stock material should be aliquoted and stored at -80°C.

10.2 Label eight tubes with #1 - 8.

10.3 Add 125 µL 1X Cell Extraction Buffer PTR into tube #2 – 8.

10.4 Prepare 50% **Control #1** by adding 62.5 µL of the 100% Stock Lysate Solution to 187.5 µL of 1X Cell Extraction Buffer PTR to tube #1. Mix thoroughly and gently.

10.5 Prepare **Control #2** by transferring 125 µL from Control #1 to tube #2. Mix thoroughly and gently.

10.6 Using the table below as a guide, repeat for Tubes #3 through #7.

10.7 **Control #8** contains no protein and is the Blank control.

ΔNote: Control lysates are supplied as a control reagent - not an absolute quantitation measure. A 3 - 4 point lysate dilution series is sufficient for this purpose.

Control #	Volume to dilute (µL)	Volume of Diluent (µL)	Lyophilized STAT5 A/B (%)
1	Step 10.4		50
2	125 µL Control #1	125	25
3	125 µL Control #2	125	12.5
4	125 µL Control #3	125	6.25
5	125 µL Control #4	125	3.13
6	125 µL Control #5	125	1.56
7	125 µL Control #6	125	0.78
8 (Blank)	N/A	125	0

11. Sample Preparation

A cell density that yields 10,000 – 40,000 cells/well is suitable for the analysis of many cell lines. The lysis buffer volume should be adjusted so that lysates are in the range of 100-500 µg/mL of protein.

11.1 Preparation of extracts from adherent cells:

11.1.1 Remove growth media and rinse adherent cells 2 times in PBS.

11.1.2 Solubilize the cells by addition of chilled 1X Cell Extraction Buffer PTR directly to the plate (for cells cultured in 96-well microplates, lyse the cells with 100 µL* of 1X Cell Extraction Buffer PTR).

*Lysis volume should be adjusted depending on the desired lysate concentration. Lysates in the range of 100 - 500 µg/mL protein are usually sufficient.

11.1.3 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.1.4 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

11.2 Preparation of extracts from non-adherent cells:

11.2.1 Collect non-adherent cells by centrifugation and resuspend at an appropriate density in RPMI containing 10% FBS. Typical centrifugation conditions for cells are 500 x g for 5 minutes at RT.

11.2.2 Return cells to a 37°C incubator for 1 - 2 hours. For certain pathways, this can allow handling-mediated pathway activation to subside. This step is optional, and depends on the activation status of your cells following re-suspension.

11.2.3 At the completion of the cell treatment, harvest cells by centrifugation and lyse with 1X Cell Extraction Buffer PTR. *

11.2.4 Alternatively, in the absence of centrifugation cells may be lysed directly with a 20% volume of 5X Cell Extraction/Enhancer Buffer PTR (e.g. for 80 µL of cells, use 20 µL of 5X Cell Extraction/Enhancer Buffer PTR).

11.2.5 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.2.6 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

*For best results, we recommend centrifugation and lysis of cells with 1X Cell Extraction Buffer PTR (11.2.3). Matrix effects may be observed in different cell media using the direct lysis approach (11.2.4). FBS may contain levels of ERK1/2 (Total) protein.

11.3 Preparations of extracts from tissue homogenates:

11.3.1 Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).

11.3.2 Homogenize 100 to 200 mg of wet tissue in 500 µL - 1 mL of chilled 1X Cell Extraction Buffer PTR. For lower amounts of tissue adjust volumes accordingly.

11.3.3 Incubate on ice for 20 minutes.

11.3.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.3.5 Transfer the supernatants into clean tubes and discard the pellets.

11.3.6 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.3.7 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

Refer to Dilution Guidelines for further instruction.

Guidelines for Dilutions of 100-fold or Greater *(for reference only; please follow the insert for specific dilution suggested)*	
100x	**10000x**
4 µl sample + 396 µl buffer (100X) = 100-fold dilution *Assuming the needed volume is less than or equal to 400 µl*	A) 4 µl sample + 396 µl buffer (100X) B) 4 µl of A + 396 µl buffer (100X) = 10000-fold dilution *Assuming the needed volume is less than or equal to 400 µl*
1000x	**100000x**
A) 4 µl sample + 396 µl buffer (100X) B) 24 µl of A + 216 µl buffer (10X) = 1000-fold dilution *Assuming the needed volume is less than or equal to 240 µl*	A) 4 µl sample + 396 µl buffer (100X) B) 4 µl of A + 396 µl buffer (100X) C) 24 µl of A + 216 µl buffer (10X) = 100000-fold dilution *Assuming the needed volume is less than or equal to 240 µl*

12. Plate preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or "edge effects" have not been observed with this assay.

13. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.

13.1 Prepare all reagents, working standards, and samples as directed in the previous sections.

13.2 Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.

13.3 Add 50 µL of all samples and standards to appropriate wells.

13.4 Add 50 µL of the Antibody Cocktail to each well.

13.5 Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.

13.6 Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid.

13.7 Add 100 µL of TMB Substrate to each well and incubate for 15 minutes in the dark on a plate shaker set to 400 rpm.

13.8 Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.

Alternative to 13.7 – 13.8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed time in the microplate reader prepared with the following settings:

Mode:	Kinetic
Wavelength:	600 nm
Time:	up to 15 min
Interval:	20 sec - 1 min
Shaking:	Shake between readings

ΔNote: A*n endpoint reading can also be recorded at the completion of the kinetic read by adding 100 µL Stop Solution to each well and recording the OD at 450nm.*

13.9 Analyze the data as described below.

14. Calculations

14.1.1 Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values.

14.1.2 Create a standard curve by plotting the average blank control subtracted absorbance value for each standard concentration (y axis) against the target protein concentration (x axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.

14.1.3 Note: Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4 parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.

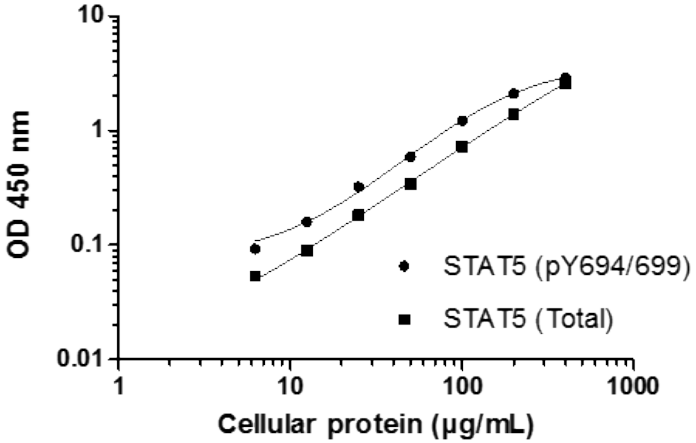
14.1.4 Determine the concentration of the target protein in the sample by interpolating the blank control subtracted absorbance values against the standard curve. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.

14.1.5 Samples generating absorbance values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance values less than that of the lowest standard should be retested in a less dilute form.

*Control Lysates are supplied as a control reagent - not an absolute quantitation measure.

15. Typical Data

Typical standard curve – data provided **for demonstration purposes only.** A new standard curve must be generated for each assay performed.



Lysate Dilution Series Measurements						
	STAT5 (pY694/699)			*STAT5 (Total)*		
Conc.	*O.D. 450 nm*		*Mean*	*O.D. 450 nm*		*Mean*
(µg/mL)	*1*	*2*	*O.D.*	*1*	*2*	*O.D.*
0.000	*0.046*	*0.045*	*0.046*	*0.048*	*0.049*	*0.049*
6.250	*0.137*	*0.140*	*0.139*	*0.103*	*0.102*	*0.103*
12.50	*0.206*	*0.207*	*0.207*	*0.140*	*0.137*	*0.139*
25.00	*0.364*	*0.376*	*0.370*	*0.230*	*0.231*	*0.231*
50.00	*0.620*	*0.650*	*0.635*	*0.383*	*0.392*	*0.388*
100.0	*1.246*	*1.289*	*1.268*	*0.781*	*0.778*	*0.780*
200.0	*2.173*	*2.148*	*2.161*	*1.406*	*1.458*	*1.432*
400.0	*2.935*	*2.972*	*2.954*	*2.623*	*2.648*	*2.636*

Figure 1. Example of a typical ERK1/2 (pT202/Y204) and ERK1/2 (Total) cell lysate dilution series. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

16. Typical Sample Values

SENSITIVITY –

The calculated minimal detectable (MDD) dose of STAT5 A/B (pY694/699) and STAT5 A/B (Total) protein in activated A431 cell extracts is approximately 5 µg/mL. Data is indicative only – the proportion of total protein that is phosphorylated is unknown.

As a guide, STAT5 A/B (pY694/699) and STAT5 A/B (Total) is detectable in activated A431 cell extracts (EGF treated) with a total cellular protein concentration < 5 µg/mL (see Figure 1).

PRECISION –

Mean coefficient of variations of interpolated values from 3 concentrations of A431 extracts within the working range of the assay.

Target		Intra-Assay	Inter-Assay
STAT5 A/B (pY694/699)	n=	6	3
	CV (%)	2.8	4.3
STAT5 A/B (Total)	n=	6	3
	CV (%)	3.9	2.1

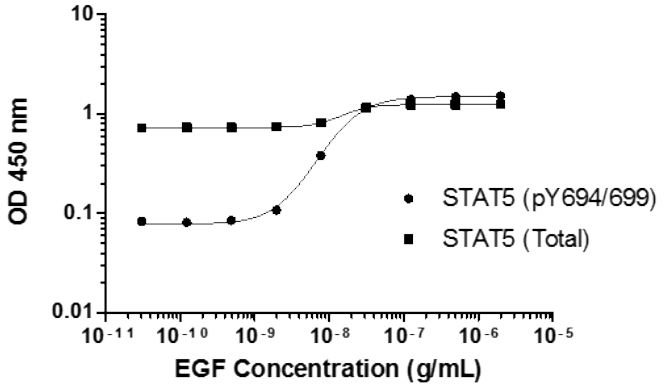


Figure 2. Induction of STAT5 A/B (pY694/699) phosphorylation in A431 cells in response to EGF treatment. A431 cells were cultured in 96-well tissue culture plates and treated (15 minutes) with a dose-range of EGF before cell lysis. Data from duplicate measurements of STAT5 A/B (pY694/699) and STAT5 (Total) are plotted.

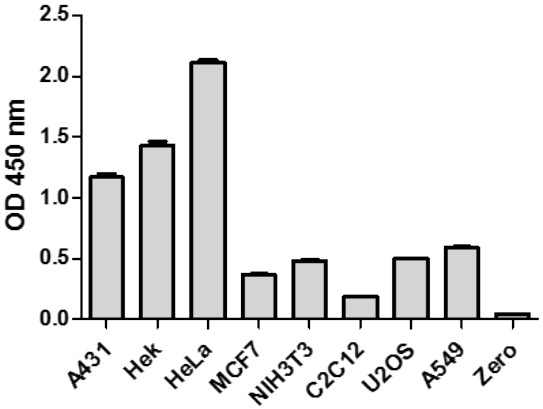


Figure 3. Cell line analysis for Total STAT5 A/B from 100 µg/mL preparations of cell extracts. Data from triplicate measurements (mean +/- SD) are plotted and compared to 1X Cell Extraction Buffer PTR (zero).

17. Assay Specificity

The STAT5 A/B (pY694/699) assay detects endogenous levels of STAT5 A and STAT5 B (GenBank Accessions NP_003143 and NP_036580) in cellular lysates, only when phosphorylated at Tyr694/699.

The STAT5 A/B assay detects endogenous levels of STAT5 A and STAT5 B (GenBank Accessions NP_003143 and NP_036580) in cellular lysates, irrespective of phosphorylation status.

18. Species Reactivity

This kit detects STAT5 A/B (pY694/699) in Human cell culture extracts. Detection in mouse and rat cells is also expected. Other species should be tested on a case-by-case basis.

Serum and plasma samples have not been tested with this kit.

Please contact our Technical Support team for more information.

19. Troubleshooting

Problem	Cause	Solution
Difficulty pipetting lysate; viscous lysate.	Genomic DNA solubilized	Prepare 1X Cell Extraction Buffer PTR (without enhancer). Add enhancer to lysate after extraction.
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low signal	Incubation times too brief	Ensure sufficient incubation times; increase to 2 or 3 hour standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Incubation times with TMB too brief	Ensure sufficient incubation time until blue color develops prior addition of Stop solution
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your reconstituted standards at -80°C, all other assay components 4°C. Keep TMB substrate solution protected from light.

20. Notes

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