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TNF-alpha Human SimpleStep ELISA® Kit

For the quantitative measurement of TNF-alpha in human serum, plasma and culture media.

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

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1. Overview

TNF-alpha in vitro SimpleStep® ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the accurate quantitative measurement of TNF-alpha protein in human serum, plasma and culture media.

The SimpleStep® ELISA employs a labeled capture and detector antibody which immunocaptures the sample analyte in solution. This entire complex (capture antibody/protein/detector antibody) is in turn immobilized in the well by immunoaffinity via the anti-tag antibody. Samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material; the TMB substrate is then added. The reaction is stopped by addition of Stop Solution which stops the color development and completes 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.

TNF-alpha, also known as cachectin or TNFSF1A, is the prototypic ligand of the TNF superfamily which plays a central role in inflammation, apoptosis, proliferation, invasion, angiogenesis, metastasis and morphogenesis. It is expressed on macrophages, endothelial, epithelial and tumor cells as a 26kDa transmembrane protein. TNF-alpha is cleaved by proteolytic processing into six chains: (1) TNF membrane form, (2) Intracellular domain 1, (3) Intracellular domain 2, (4) C-domain 1, (5) C-domain 2 and (6) TNF soluble form. Signaling from TNF-alpha differs depending on the type of ligand initiating the signaling event (intracellular, membrane or soluble). As an example, the membrane form of TNF-alpha appears to mediate anti-tumorigenic therapeutic responses whereas the soluble ligand is linked to inflammation and proliferation.

2. Protocol Summary

Remove appropriate number of antibody coated well strips. Equilibrate all reagents to room temperature. 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
TNF-alpha Capture Antibody (Lyophilized)	2 Vials	4°C
TNF-alpha Detector Antibody (Lyophilized)	2 Vials	4°C
TNF-alpha Lyophilized Recombinant Protein	2 Vials	4°C
Antibody Diluent 5BI	1 x 6 mL	4°C
10X Wash Buffer PT	20 mL	4°C
TMB Substrate	12 mL	4°C
Stop Solution	12 mL	4°C
Sample Diluent NS	50 mL	4°C
Sample Diluent 50BP	1 x 20 mL	4°C
Pre-Coated 96 Well Microplate (12 x 8 well strips)	96 Wells	4°C
Plate Seal	1	4°C

7. Materials Required, Not Supplied

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

- 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 KH_2PO_4, 8 mM Na_2HPO_4, 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.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

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.
- 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.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation.
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.
- When generating positive control samples, it is advisable to change pipette tips after each step.
- The provided 5X Cell Extraction Buffer contains phosphatase inhibitors. Protease inhibitors can be added if required.

- The provided 50X Cell Extraction Enhancer Solution may precipitate when stored at + 4ºC. To dissolve, warm briefly at + 37ºC and mix gently. The 50X Cell Extraction Enhancer Solution can be stored at room temperature to avoid precipitation.
- To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. 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 provided 20X formulations.

9.1 1X Wash Buffer PT

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.

9.2 20X TNF-alpha Capture antibody

To reconstitute the lyophilized TNF-alpha Capture antibody, centrifuge the vial at 10,000 x *g* for 2 minutes and then add 150 µL of nanopure water per vial; incubate at room temperature for 10 minutes on rotator ensuring the material is fully resuspended.

For long term storage, add to the 20X TNF-alpha capture antibody 150 µL of 100% glycerol to make a 10X TNF-alpha capture antibody stock and freeze at -20°C. Avoid repeat freeze/thaw cycles.

9.3 20X TNF-alpha Detector antibody

To reconstitute the lyophilized TNF-alpha Detector antibody, centrifuge the vial at 10,000 x *g* for 2 minutes and then add 150 µL of nanopure water per vial; incubate at room temperature for 10 minutes on rotator ensuring the material is fully resuspended.

For long term storage, add to the 20X TNF-alpha Detector antibody 150 µL of 100% glycerol to make a 10X TNF-alpha Detector antibody stock and freeze at -20°C. Avoid repeat freeze/thaw cycles.

9.4 Antibody Cocktail

Prepare Antibody Cocktail by diluting in the 20X Capture Antibody and the 20X Detector Antibody in Antibody Diluent 5BI. To make 3 mL of the Antibody Cocktail combine 150 µL 20X Capture Antibody and 150 µL 20X Detector Antibody with 2.7 mL Antibody Diluent. Mix thoroughly and gently.

ΔNote: If using the 10X TNF-alpha capture and detector antibodies, adjust volumes accordingly.

10. Standard Preparation

- Prepare serially diluted standards immediately prior to use.
- 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 For **serum, plasma heparin & EDTA or cell culture media samples** follow these instructions:

10.1.1 Reconstitute TNF-alpha standard sample by adding 100 µL water by pipette. Mix thoroughly and gently. Hold at room temperature for 10 minutes. This is the 40,000 pg/mL **Stock Standard** Solution.

10.1.2 Label eight tubes, standards 1-8.

10.1.3 Add 380 µL Sample Diluent NS into **Standard #1** and 150 µL Sample Diluent NS into **Standard #2-8.**

10.1.4 To prepare **Standard #1**, add 20 µL of the **Stock Standard** into tube #1 and mix gently.

10.1.5 To prepare **Standard #2**, add 150 µL of the **Standard #1** into tube #2 and mix gently.

10.1.6 Repeat for tubes #3 through #8.

10.1.7 Using the table below as a guide, prepare subsequent serial dilutions. Standard #8 contains no protein and is the Blank control.

Standard #	Volume to dilute (µL)	Volume Diluent N (µL)	Human TNF-alpha (pg/mL)
1	Stock	20	2,000
2	150 µL Standard #1	150	1,000
3	150 µL Standard #2	150	500
4	150 µL Standard #3	150	250
5	150 µL Standard #4	150	125
6	150 µL Standard #5	150	62.5
7	150 µL Standard #6	150	31.25
8 (Blank)	N/A	150	0

10.2 For **plasma citrate samples** follow these instructions:

10.2.1 Reconstitute TNF-alpha standard sample by adding 100 µL water by pipette. Mix thoroughly and gently. Hold at room temperature for 10 minutes. This is the 40,000 pg/mL **Stock Standard** Solution.

10.2.2 Label eight tubes, standards 1-8.

10.2.3 Add 390 µL Sample Diluent 50BP into **Standard #1** and 150 µL Sample Diluent 50BP into **Standard #2-8.**

10.2.4 To prepare **Standard #1**, add 10 µL of the **Stock Standard** into tube #1 and mix gently.

10.2.5 To prepare **Standard #2**, add 150 µL of the **Standard #1** into tube #2 and mix gently.

10.2.6 Repeat for tubes #3 through #8.

10.2.7 Using the table below as a guide, prepare subsequent serial dilutions. Standard #8 contains no protein and is the Blank control.

Standard #	Volume to dilute (µL)	Volume Diluent (µL)	Human TNF-alpha (pg/mL)
1	Stock	10	1,000
2	150 µL Standard #1	150	500
3	150 µL Standard #2	150	250
4	150 µL Standard #3	150	125
5	150 µL Standard #4	150	62.5
6	150 µL Standard #5	150	31.2
7	150 µL Standard #6	150	15.63
8 (Blank)	N/A	150	0

11. Sample Preparation

TYPICAL SAMPLE DYNAMIC RANGE	
Sample Type	Range (%)
Human Serum	10 - 100
Human Plasma Citrate	10 - 100
Human Plasma Heparin	10 - 100
Human Plasma EDTA	10 – 100
Conditioned Media	10 - 100

11.1 Plasma:

Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x *g* for 10 minutes. Dilute plasma heparin and EDTA into Sample Diluent NS and assay. Dilute plasma citrate in Sample Diluent 50BP. Store un-diluted plasma samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

11.2 Serum:

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x *g* for 10 minutes and collect serum. Dilute samples into Sample Diluent NS and assay. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.

11.3 Cell Culture Supernatants:

Centrifuge cell culture media at 2,000 x *g* for 10 minutes to remove debris. Collect supernatants and dilute to desire concentration in Sample Diluent NS and assay. Store samples at -20°C or below. Avoid repeated freeze-thaw cycles.

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 sample or standard 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 10 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

Note that an 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 450 nm.

13.9 Analyze the data as described below.

14. Calculations

14.1 Subtract the average zero standard from all readings.

14.2 Average the duplicate readings of the positive control dilutions and plot against their concentrations.

14.3 Draw the best smooth curve through these points to construct the standard curve.

ΔNote: Most microplate reader software or graphing software can 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.

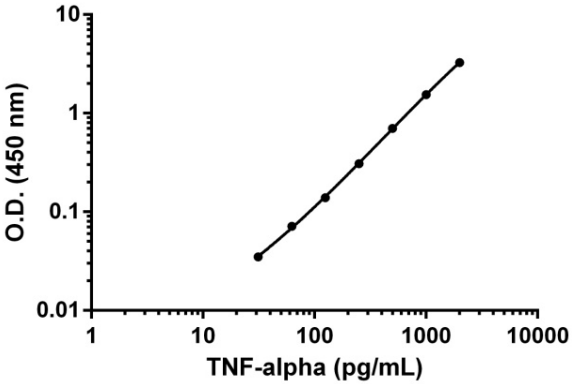
14.4 Interpolate protein concentrations for unknown samples from the standard curve plotted.

14.5 Samples producing signals 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.

14.6 Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.

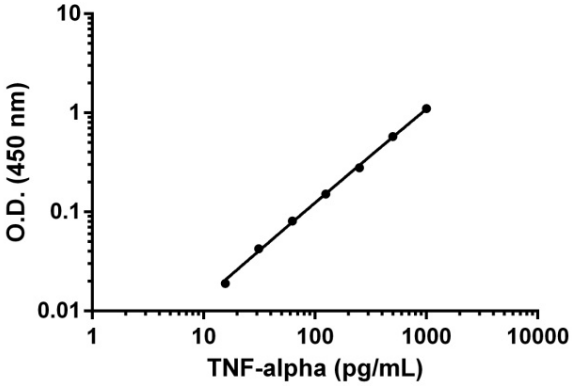
15. Typical Data

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



Standard Curve Measurements			
Conc. (pg/mL)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.061	0.067	0.064
31.2	0.101	0.097	0.099
62.5	0.132	0.139	0.136
125	0.205	0.201	0.203
250	0.375	0.369	0.372
500	0.768	0.767	0.768
1,000	1.593	1.624	1.608
2,000	3.322	3.339	3.330

Figure 1. Example of TNF-alpha standard curve. The TNF-alpha standard curve was prepared as described in Section 10 in Sample Diluent NS. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.



Standard Curve Measurements			
Conc. (pg/mL)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.051	0.051	0.051
15.63	0.083	0.070	0.077
31.2	0.096	0.091	0.094
62.5	0.133	0.132	0.132
125	0.204	0.202	0.203
250	0.327	0.335	0.331
500	0.640	0.617	0.629
1,000	1.158	1.160	1.159

Figure 2. Example of TNF-alpha standard curve. The TNF-alpha standard curve was prepared as described in Section 10 in Sample Diluent 50BP. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

16. Calibration

This immunoassay is calibrated against a highly purified human TNF-alpha. The NIBSC/WHO unclassified purified human TNF-alpha preparation 88/786 was evaluated in this kit.

The dose response curve of the unclassified standard 88/786 parallels the SimpleStep standard curve. To convert sample values obtained with the SimpleStep human TNF-alpha kit to approximate NIBSC 88/786 International units, use the equation below.

NIBSC (88/786) approximate value (IU/mL) = 0.083 x SimpleStep™ human TNF-alpha value (pg/mL).

17. Typical Sample Values

SENSITIVITY –

The calculated minimal detectable dose (MDD) is 14 pg/mL. The MDD was determined by calculating the mean of zero standard replicates (n=27) and adding 2 standard deviations then extrapolating the corresponding concentration.

RECOVERY –

Three concentrations of TNF-alpha were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

Sample Type	Average % Recovery	Range (%)
Human Serum	99.50	80 – 119
Human Plasma - Citrate	93.09	83.5 – 99
Human Plasma - EDTA	102.00	95.7 – 110
Human Plasma - Heparin	98.00	91 – 106
Cell Culture Media	100.97	95 - 109

Linearity of Dilution

Recombinant TNF-alpha was spiked into the following biological samples and diluted in a 2-fold dilution series in Sample Diluent NS (for serum, plasma EDTA, plasma heparin and cell culture media) or Sample Diluent 50BP (for plasma citrate).

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

Dilution Factor	Interpolated value	100 % Human Serum	100 % Human Plasma (Citrate)	100 % Human Plasma (EDTA)	100% Human Plasma (Heparin)	100% Culture Media
Undiluted	pg/mL	867	990.4	1032.6	840.2	1107.5
	% Expected value	**100**	**100**	**100**	**100**	**100**
2	pg/mL	454.2	489.6	609.5	525.8	580.7
	% Expected value	**100**	**104**	**113**	**125**	**105**
4	pg/mL	236.8	225.3	305.9	282	291.09
	% Expected value	**109**	**95**	**119**	**134**	**105**
8	pg/mL	124	106.7	142.8	136.7	125.75
	% Expected value	**135**	**90**	**130**	**130**	**91**

PRECISION –

Mean coefficient of variations of interpolated values from 3 concentrations of PHA stimulated PBMC media within the working range of the assay.

	Intra-Assay	Inter-Assay
n=	8	3
CV (%)	2.3	5

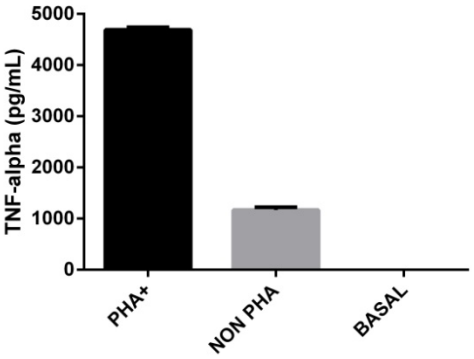


Figure 3. Specificity of TNF-alpha signal on stimulated and non stimulated media supernatants. Human PBMCs were cultured in RPMI supplemented with 10% fetal calf serum, 2mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were cultured for 2 days at 37°C in the presence or absence of PHA. The concentrations of TNF-alpha were interpolated from the calibration curve and corrected for sample dilution. The mean TNF-alpha concentration was determined to be undetectable in basal media, 1.2 ng/mL in unstimulated PMBC supernatants and 4.7 ng/mL in stimulated PBMC supernatants.

Ten individual healthy donors were evaluated for the presence of TNF-alpha in serum using this assay. All samples had undetectable levels of TNF-alpha.

18. Assay Specificity

This assay recognizes native and recombinant human TNF-alpha.

The recombinant human proteins listed below were prepared at 50 ng/mL in Sample Diluent NS and assayed for cross reactivity. No significant cross-reactivity was observed with a mean OD deviation from background of 0.003.

IFN-gamma	IL-6	EGF
IL-1 alpha	IL6R	G-SCF
IL1RA	IL-8	GM-CSF
IL-2	IL-17a	SCF
IL-4	IL12p70	RANTES
CCL20	CCL24	CRP

Recombinant TNF-alpha control was assayed at 500 pg/mL in the presence and absence of 50 ng/mL of the recombinant proteins listed above to determine interference. Recovery of TNF-alpha was observed on average at 101% with a standard deviation of 3.2%.

19. Species Reactivity

This kit recognizes both native and recombinant human TNF-alpha protein in the plasma, serum and culture media samples.

It does not cross-react with mouse TNF-alpha.

Please contact our Technical Support team for more information.

20. Troubleshooting

Problem	Reason	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.

21. Notes

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