เอกสารแนบที่ 3

Quantikine®

Rat IL-1β/IL-1F2 Immunoassay

Catalog Number RLB00
SRLB00
PRLB00

For the quantitative determination of rat interleukin 1 beta (IL-1β) concentrations in cell culture supernates, rat serum, and rat EDTA plasma.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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INTRODUCTION

The interleukin 1 (IL-1) family of proteins consists of IL-1α, IL-1β and the IL-1 receptor antagonist. IL-1α and IL-1β bind to the same cell surface receptors and share biological functions. With the exception of skin keratinocytes, some epithelial cells, and certain cells of the central nervous system, IL-1 is not produced by unstimulated cells of healthy individuals. However, in response to stimuli such as those produced by inflammatory agents, infections, or microbial endotoxins, a dramatic increase in the production of IL-1 by macrophages and various other cell types is seen (1 - 4).

IL-1α and IL-1β are structurally related polypeptides that show approximately 25% homology at the amino acid level (2). Both are synthesized as 31 kDa precursors that are subsequently cleaved into proteins with molecular weights of approximately 17.5 kDa (5, 6). Neither IL-1α nor IL-1β contains a typical hydrophobic signal peptide sequence (7 - 9), but current evidence suggests that these factors can be secreted by non-classical pathways (10, 11). A large proportion of IL-1α is retained intracellularly in its precursor form (3). A portion of this unprocessed IL-1α is transported to the cell surface and remains associated with the cell membrane (1, 3, 12). The membrane-bound, unprocessed IL-1α is apparently biologically active, acting in a paracrine fashion on adjacent cells having IL-1 receptors (1, 3, 4). The precursor form of IL-1β, unlike the IL-1α precursor, shows little or no biological activity in comparison to the 17.5 kDa processed form (11, 13, 14). Intracellular IL-1β consists exclusively of the 31 kDa precursor form (6). Extracellular IL-1β consists of a mixture of both unprocessed and mature IL-1β. These results indicate that processing takes place subsequent to secretion and is not tightly coupled to secretion (6, 10, 11, 15). The specific protease apparently responsible for the processing of IL-1β, designated interleukin-1β-converting enzyme (ICE), has been described (15).

IL-1α and IL-1β exert their effects by binding to specific receptors. Two distinct receptor types have been isolated that bind both forms of IL-1. An 80 kDa membrane bound receptor protein (IL-1 RI) has been isolated from T cells, fibroblasts, keratinocytes, endothelial cells, synovial lining cells, chondrocytes, and hepatocytes (1, 3, 16). The IL-1 RI has been cloned from mouse and human cells (17) and found to be a member of the Ig super family. A second type of IL-1 receptor (IL-1 RII) has been found on B cells, neutrophils, and bone marrow cells (1, 3). This receptor has an apparent molecular weight of about 68,000 and is also a member of the Ig super family. The two IL-1 receptor types show approximately 28% homology in their extracellular domains, but differ significantly in that the type II receptor has a cytoplasmic domain of only 29 amino acid residues, whereas the type I receptor has a cytoplasmic domain of 213 amino acid residues (1, 17). Recently, a third type of IL-1 receptor, referred to as IL-1 receptor accessory protein (IL-1 RAcP), has been cloned from murine cells (18). While IL-1 RAcP does not appear to bind IL-1 directly, the protein is required for transduction of biological responses and forms a complex with IL-1 RI that binds IL-1 with higher affinity than IL-1 RI alone. IL-1 RII does not appear to be involved in IL-1 signaling and may function as a "decoy" receptor that attenuates IL-1 function (19). Soluble forms of both IL-1 RII and IL-1 RI have been detected in human plasma, synovial fluids and the conditioned media of several human cell lines (20, 21). In addition, it has been found that vaccinia and cowpox viruses encode IL-1 binding proteins that resemble soluble IL-1 RII (22).

IL-1 possesses a wide variety of biological activities and plays a central role in mediating immune and inflammatory responses. Although normal production of IL-1 is obviously critical for initiation of normal host responses to injury and infection, inappropriate or prolonged production of IL-1 has been implicated as playing a role in the production of a variety of pathological conditions including sepsis, rheumatoid arthritis, inflammatory bowel disease, acute and chronic myelogenous leukemia, insulin-dependent diabetes mellitus, and atherosclerosis (1, 3, 4).

The Quantikine Rat IL-1β Immunoassay is a 4.5 hour solid phase ELISA designed to measure rat IL-1β in cell culture supernates, serum, and EDTA plasma. It contains recombinant rat IL-1β and antibodies raised against recombinant rat IL-1β. This immunoassay has been shown to quantitate the recombinant rat factor accurately.

It has been reported that ELISA kits calibrated using mature IL-1β as a standard will detect, but considerably underestimate, the unprocessed IL-1β precursor present in samples (23, 24). While the rat IL-1β precursor has not been tested in this immunoassay kit, it is possible that this kit may also underestimate the rat precursor IL-1β in samples. Nevertheless, in biological samples other than cell lysates, the precursor form of IL-1β (which is not biologically active) is usually not the predominant form of IL-1β. Therefore, results obtained using the Quantikine Rat IL-1β Immunoassay kit should provide a useful measure of the levels of active rat IL-1β present in biological fluids.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for rat IL-1β has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any rat IL-1β present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat IL-1β is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of rat IL-1β bound in the initial step. The sample values are then read off the standard curve.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested, the possibility of interference cannot be excluded.

PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- For best results, pipette reagents and samples into the center of each well.
- It is recommended that the samples be pipetted within 15 minutes.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.

MATERIALS PROVIDED

Description	Part #	Cat. # RLB00	Cat. # SRLB00
Rat IL-1β Microplates - 96 well polystyrene microplates (12 strips of 8 wells) coated with polyclonal antibody specific for rat IL-1β.	890539	2 plates	6 plates
Rat IL-1β Conjugate - 23 mL/vial of a polyclonal antibody against rat IL-1β conjugated to horseradish peroxidase with preservatives.	890540	1 vial	3 vials
Rat IL-1β Standard- 4 ng/vial of recombinant rat IL-1β in a buffered protein base with preservatives; lyophilized.	890541	3 vials	9 vials
Rat IL-1β Control - Recombinant rat IL-1β in a buffered protein base with preservatives; lyophilized. The concentration range of rat IL-1β after reconstitution is shown on the vial label. The assayed value of the Control should be within the range specified on the label.	890542	3 vials	9 vials
Assay Diluent RD1-21 - 12 mL/vial of a buffered protein solution with preservatives.	895215	1 vial	3 vials
Calibrator Diluent RD5Y - 21 mL/vial of a buffered protein solution with preservatives.	895201	2 vials	6 vials
Wash Buffer Concentrate - 50 mL/vial of a 25-fold concentrated solution of a buffered surfactant with preservative.	895024	1 vial	3 vials
Color Reagent A - 12 mL/vial of stabilized hydrogen peroxide.	895000	1 vial	3 vials
Color Reagent B - 12 mL/vial of stabilized chromogen (tetramethylbenzidine).	895001	1 vial	3 vials
Stop Solution - 23 mL/vial of a diluted hydrochloric acid solution.	895174	1 vial	3 vials
Plate Covers - Adhesive strips.	640197	8 strips	24 strips

RLB00 contains sufficient materials to run ELISAs on two 96 well plates.
SRLB00 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PRLB00). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

STORAGE

Unopened Kit	Store at 2 - 8° C. Do not use beyond kit expiration date.	
Opened/ Reconstituted Reagents	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Calibrator Diluent RD5Y	
	Assay Diluent RD1-21	
	Rat IL-1β Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	
	Rat IL-1β Standard (2000 pg/mL)	Discard within 8 hours of reconstitution. Use a new standard and control for each assay.
	Rat IL-1β Control	
	Microplate Wells	Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 1000 mL graduated cylinders.
- Test tubes for dilution.

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Serum - Allow blood samples to clot for 2 hours at room temperature or overnight at 2 - 8° C before centrifuging for 20 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA as an anticoagulant. Centrifuge for 20 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples ≤ -20° C. Avoid repeated freeze-thaw cycles.

Note: *Grossly hemolyzed or lipemic samples may not be suitable for measurement of rat IL-1β with this assay.*

SAMPLE PREPARATION

Rat serum and rat EDTA plasma samples require a 3-fold dilution into Calibrator Diluent RD5Y. A suggested 3-fold dilution is 50 µL sample + 100 µL Calibrator Diluent RD5Y.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

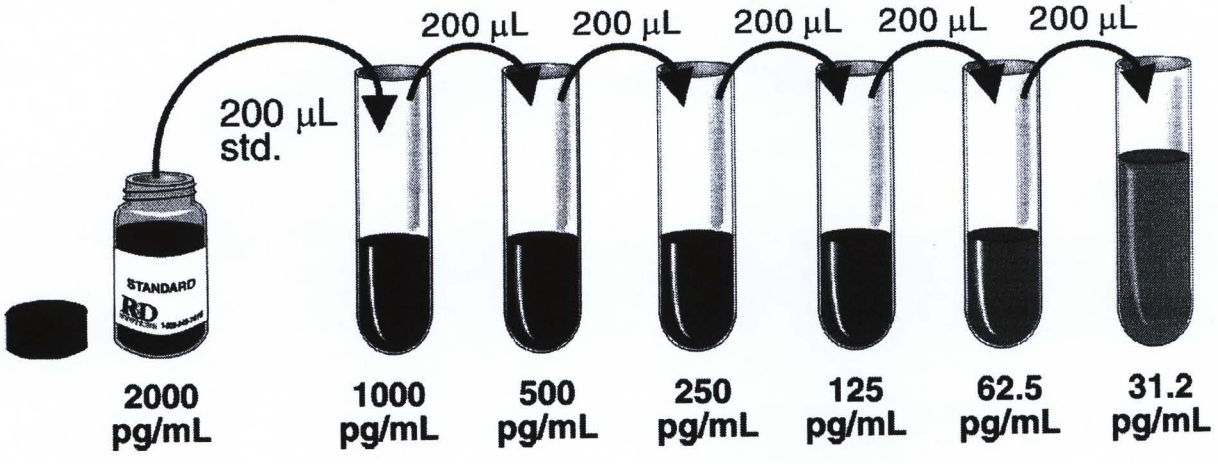
Rat IL-1β Kit Control - Reconstitute the Kit Control with 1.0 mL deionized or distilled water. Assay the Control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 25 mL Wash Buffer Concentrate to deionized or distilled water to prepare 625 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

Rat IL-1β Standard - Reconstitute the Rat IL-1β Standard with 2.0 mL of Calibrator Diluent RD5Y. Do not substitute other diluents. This reconstitution produces a stock solution of 2000 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 200 µL of Calibrator Diluent RD5Y into each tube. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted rat IL-1β Standard serves as the high standard (2000 pg/mL). Calibrator Diluent RD5Y serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and control be assayed in duplicate.

1. Prepare all reagents, standard dilutions, control, and samples as directed by the previous sections.

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

3. Add 50 µL of Assay Diluent RD1-21 to each well.

4. Add 50 µL of Standard, Control, or sample* per well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.

5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 100 µL of Rat IL-1β Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 5.

8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**

9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Serum/Plasma samples require a 3-fold dilution as directed in the Sample Preparation section.

PROCEDURE SUMMARY AND CHECKLIST

1. ☐ Bring all reagents to room temperature.
   ☐ Prepare reagents and samples as instructed.
   ☐ Return unused components to storage temperature as indicated in the instructions.

2. ☐ Add 50 µL Assay Diluent to each well.

3. ☐ Add 50 µL Standard, Control, or sample* to each well.
   ☐ Tap plate gently for one minute.
   ☐ Cover the plate and incubate for 2 hours at room temperature.

4. ☐ Aspirate and wash each well five times.

5. ☐ Add 100 µL Conjugate to each well.
   ☐ Cover the plate and incubate for 2 hours at room temperature.

6. ☐ Aspirate and wash each well five times.

7. ☐ Add 100 µL Substrate Solution to each well. Incubate 30 minutes at room temperature. **Protect from light.**

8. ☐ Add 100 µL Stop Solution to each well.

9. ☐ Read Optical Density at 450 nm (correction wavelength set at 540 nm or 570 nm).

*Serum/Plasma samples require a 3-fold dilution.

CALCULATION OF RESULTS

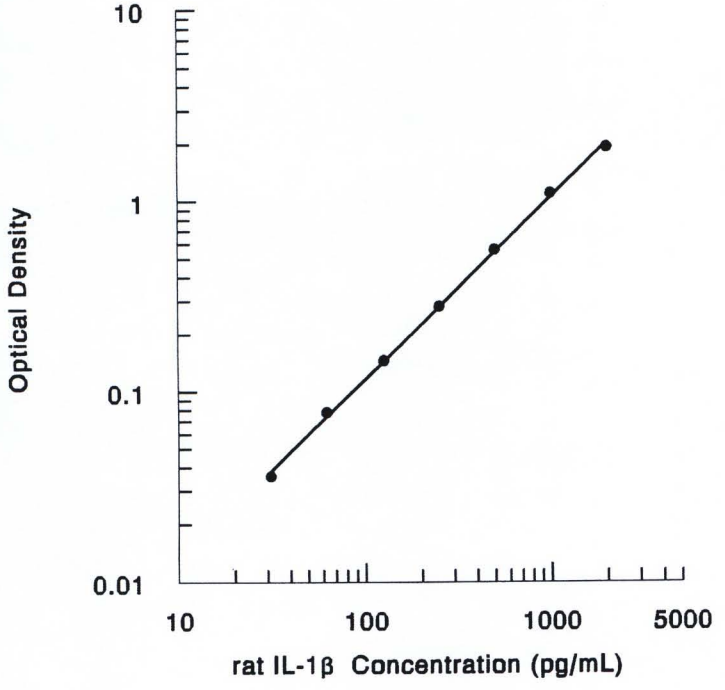
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Create a standard curve by reducing the data using computer software capable of generating a log-log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. This procedure will produce an adequate but less precise fit of the data.

Because serum and plasma samples have been diluted prior to the assay, the measured concentrations must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.035 0.034	0.034	—
31.2	0.070 0.069	0.070	0.036
62.5	0.111 0.113	0.112	0.078
125	0.177 0.182	0.180	0.146
250	0.322 0.310	0.316	0.282
500	0.592 0.593	0.592	0.558
1000	1.143 1.135	1.139	1.105
2000	1.962 1.960	1.961	1.927

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty assays to assess inter-assay precision.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	83.4	253	610	82.3	249	626
Standard deviation	7.3	9.9	24	4.7	10.3	27
CV (%)	8.8	3.9	3.9	5.7	4.1	4.4

RECOVERY

The recovery of rat IL-1β spiked to three levels in five samples throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture supernates (n = 5)	100	89 - 110%
Rat serum* (n = 5)	108	97 - 120%
Rat EDTA plasma* (n = 5)	98	89 - 111%

*Rat serum and plasma samples were first diluted 3-fold, as directed by the Sample Preparation.

SENSITIVITY

The minimum detectable dose of rat IL-1β is typically less than 5 pg/mL.

The minimum detectable dose was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

LINEARITY

To assess the linearity of the assay, five or more samples spiked with various concentrations of rat IL-1β in each matrix were diluted with Calibrator Diluent RD5Y and then assayed.

Sample	Dilution	Observed (pg/mL)	Expected (pg/mL)	Observed / Expected x 100
Cell Culture Supernates	spiked	1872		
	1/2	1078	936	115
	1/4	522	468	112
	1/8	251	234	107
	1/16	122	117	104
Rat Serum*	spiked	1026		
	1/2	541	513	105
	1/4	270	256	105
	1/8	138	128	108
	1/16	66	64	103
Rat EDTA Plasma*	spiked	819		
	1/2	436	409	107
	1/4	218	204	107
	1/8	112	102	110
	1/16	54	51	107

*Rat serum and plasma samples were first diluted 3-fold, as directed by the Sample Preparation.

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant rat IL-1β produced at R&D Systems. This recombinant rat IL-1β contains 153 amino acid residues and has a predicted molecular mass of 17 kDa.

Based on total amino acid analysis, the absorbance of a 1 mg/mL solution of *E. coli*-expressed recombinant rat IL-1β at 280 nm was determined to be 0.64 A.U.

SAMPLE VALUES

Cell Culture Supernates -
Three Wistar rats were given an intraperitoneal injection of 100 μg/kg of LPS. All rats were killed 4 hours after receiving LPS. Lungs and spleens were harvested and broken into individual cells by using a Daunce homogenizer.

Rat lung conditioned media was collected after culturing in 60 mL of RPMI supplemented with 10% fetal bovine serum for 18 hours. The cell culture supernate was assayed for rat IL-1β and measured 1124 pg/mL.

Rat spleen conditioned media was collected after culturing in 60 mL of RPMI supplemented with 10% fetal bovine serum and stimulated with 1 μg/mL of LPS for 18 hours. The cell culture supernate was assayed for rat IL-1β and measured 3752 pg/mL.

Serum - Forty individual rat serum samples were evaluated for detectable levels of rat IL-1β in this assay. Thirty-seven samples read below the lowest standard, 31.2 pg/mL. Three samples read 41, 51, and 123 pg/mL, respectively.

Plasma - Twenty individual rat EDTA plasma samples were evaluated for detectable levels of rat IL-1β in this assay. Nineteen samples read less than the lowest standard, 31.2 pg/mL. One sample read 137 pg/mL.

SPECIFICITY

This assay recognizes both recombinant and natural rat IL-1β. The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5Y and assayed for cross-reactivity. Preparations of the following factors prepared at 50 ng/mL in a mid-range rat IL-1β control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant rat:
CINC-1
GDNF
IFN-γ
IL-1α
IL-1 RI
IL-1ra
IL-2
IL-4
β-NGF
PDGF-BB
TNF-α

Recombinant mouse:
IL-1α
IL-1ra

Recombinant human:
IL-1 RI
IL-1 RII
IL-1ra

Some cross-reactivity was observed with the following:

Recombinant Factor	Concentration Tested (pg/mL)	Observed Value (pg/mL)	% Cross-reactivity
mouse IL-1β	50,000	871	1.7
human IL-1β	50,000	816	1.6

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NOTES