EXHIBIT 12

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LABORATORY NOTEBOOK

No 717

AMGEN, INC.

LIN
EXHIBIT
117

AM 17 030435
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SUBJECT TO PROTECTIVE ORDER

FRITSCH v. LIN
INTER NOS 102,096
102,097, 102,334
LIN DOC NO L01112

TRIAL EXHIBIT
2118
97-10814 WGY

Exhibit: 224 Date: 12-1-99
Witness: Joan Egrie
ERNEST M SANCHEZ: RPR/CSR

NOTEBOOK NO. 717
ISSUED TO Jeri Lane
ON 4/16 19 83
DEPARTMENT
RETURNED 19

SCIENTIFIC NOTEBOOK CO.
5007 WEST DONNA DRIVE
STEVENSVILLE, MICHIGAN 49127

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INTER NOS 102,096
102,097, 102,334
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TITLE Western of Native EndoF + Neuraminidase Treated CAS F CHO GSComplex? [illegible]

Project No.
Book No.
15

From Page No.

5/7/84L

Two gels will be transferred in a western format - the First will contain 3 types of material - Genes crude epo, CAS cell epo, and CHO epo. Each of these types will be examined untreated or after treatment with neuraminidase or EndoF. The second gel will examine a variety of odds and ends - can we use tricksach for the 1st Ab? and extract of CH without creating a huge background? Does mark's B. subtilis extract contain proteinases which will degrade epo? (He needs to know this so he can determine how to modify B subtilis to express epo) Run KB2 again to see if it's still slightly larger? Test 3 yeast samples from Steve F for expression of EPO

Enzymatic Treatment and Immunoprecipitation of Samples for Gel 1

① Neuraminidase treatment

A. CHO material - 10 ml of line 3 material
CAS material - 350 λ of a combination of 2 day and 3 day 10x concentrated samples

Spike each sample w/ 20,000 cpm of I-125 epo

B. Add 50 λ 1M KoAc pH 5.3 ~~[illegible]~~ 30 pH has dropped and sample is bright yellow

Add 100 λ of 10 U/ml neuraminidase incubate at 37° for 4 hrs.

C. Genes crude material

17 λ of Genes crude (~~[illegible]~~ (4 λ ng) spike w/ 24,000 cpm of
100 λ of 50mm KoAc pH 5.3 I-125 epo
15 λ of PBS + 1% BSA

Add 5 λ of 10 U/ml neuraminidase 4 hrs at 37°

D. To stop treatment:

Genes crude - add 2x LSB - boil and freeze until ready to load gel

CHO and CAS material - Add 50 λ of Tris pH 9.0 to ↑ pH so that color is bright red

Set up immunoprecipitation as described in the next section

FRITSCH v. LIN
INTER NOS 102,096
102,097, 107,334
LIN DOC NO 101114

AM 17 030437
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To Page No.

Witnessed & Understood by me, | Date | Invented by | Date
Recorded by

Project No.
Book No.
TITLE

16

From Page No.

② Immunoprecipitation - All COS cell and CHO cell samples must be concentrated by an Ab precipitation

CHO - neuraminidase treated material plus two new tubes each containing 1ml of lot 3 material

COS - neuraminidase treated material plus two new tubes each containing 38ml of a combination of 7 day and 8 day 10X concentrated material

Spike unlabeled samples w/ 20,000 cpm of I-125 epo
Add 9μl of SD 264 #3059 T.b.3 to each tube
overnight at 4°
Collect w/ 50λ of a 1:5 protein A sepharose
= Wash as usual
Count.

Extract the neuraminidase treated samples and one new tube of each material (untreated group) with 80λ of 3x LSB. Boil. Pellet. Remove supernatant. Calculate recovery

The remaining COS cell tube and CHO cell tube will be digested w/ Endo F. They should be extracted w/ 70λ of 0.1M Tris pH 6.8 containing 0.2% SDS. Remove sup. Calculate recovery

CHO mat'l	cpm immppt	cpm in extracted pellet	cpm in extracted sup	% in sup
No treatment	2723	626	2013	
neuraminidase	3758	1036	2494	
Endo F	3144	833	2178	
COS mat'l				
No treatment	5144	1421	3469	
neuraminidase	4396	821	3257	
Endo F	4227	1066	3071	

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FRITSCH v. LIN
INTER NOS 102,096
102,097, 102,334
LIN DOC NO L01115

To Page No.

Witnessed & Understood by me, | Date | Invented by | Date
Recorded by

TITLE
Project No.
Book No.

From Page No.

③ Endo F treatment -

A. Adjust COS matl'e sup 70λ and CHO matl'e sup 70λ as follows:
Add 14λ βME (1% final)
14λ 0.5 M EDTA (50mm final)
35λ 4% NP40 (1% final)
15λ H_2O
1λ PMSF
2.5λ Trasylol
1λ endo F enzyme
140λ total volume
Incubate at 37° for 4hrs

B. Genes crude material:
60λ Endo F buffer
17λ 1110 genes crude epo (400ng)
20,000 cpm of I125 epo
5λ trasylol
1λ Endo F enzyme
Incubate at 37° for 4hrs

Add 1/10 volume of 10x LSB Boil. Ready to load on Gel.

Immunoprecipitation and pre treatment of samples for Gel #2

① precipitate w/ tachisorb

① 13λ 1110 genes crude (300ng EPO)
5λ RC-204 #3089 + b.3
15,354 cpm of I-125 epo.

② 13λ 1110 genes crude (300ng epo)
10λ RC-204 #3089 + b.3
15,354 cpm of I-125 epo

Incubate overnight at 4° Add 200λ tachisorb. Process as usual. Extract w/ 3X LSB. Calculate recovery.

To Page No.

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Recorded by

AM 17 030439
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SUBJECT TO PROTECTIVE ORDER

FRITSCH v. LIN
INTR NOS 102,096
102,097, 102,334
LIN DOC NO L01116

Project No.______

18 Book No.______ TITLE ______

From Page No.___

② Kirin Sample –
Dissolve 5 mg of KB phenyl-sepharose peak #3 lyophilized material in 0.5 ml PBS
Take O.D of a 1:20 dilution
A_{278} = 10 OD/ml
Suzuki says 1 A_{278} = 2-3 u epo

Take 0.5 ml dissolved mat'l
Add 9λ 8C 204 3089 #63
15λ I-125 epo containing 15,354 cpm (4/24/84 iodination)
overnight at 4°
Add 80λ of a 1:5 of protein A-sepharose
Process as usual calculate recovery

	cpm	cpm ext. before	cpm ext. sup	% in sup
Yachisuka ①	* 912	195	746	
Yachisuka ②	3655	436	2822	
Kirin	880	241	639	

Dissolve 5 mg of KB's phenyl sepharose peak #3 lyophilized material in 0.5 PBS.
Take OD of 1:20 diln.
A_{278} = 10 OD/ml
Suzuki said 1 A_{278} has 2-3 u EPO
∴ Take 0.5 ml + add 9λ 8C204 3089 B3
+ 15λ ^{125}I EPO (896 cpm/λ) 4/24/84 iodination
Ppt c 80λ PAS 1:5 etc.
Need to take cpm of :1λ EPO for this time

* No idea why the immunoppt is so low

③ B. subtilis samples
60λ B subtilis extract
8.5λ 1:10 crude epo (200ng)
Incubate 37° for 1hr
Extract w/ LSB Ready to load

All other samples loaded on either gel required no pretreatment. They had LSB added and boiled – previously prepared samples were again briefly boiled. Dye & Redu added.
Loaded on 2 12.5% polyacrylamide gels and run overnight at 35 V

TRITSCH V. LIN
INTER NOS 102,096
102,097, 102,334
LIN DDC NO 101117

To Page No.___

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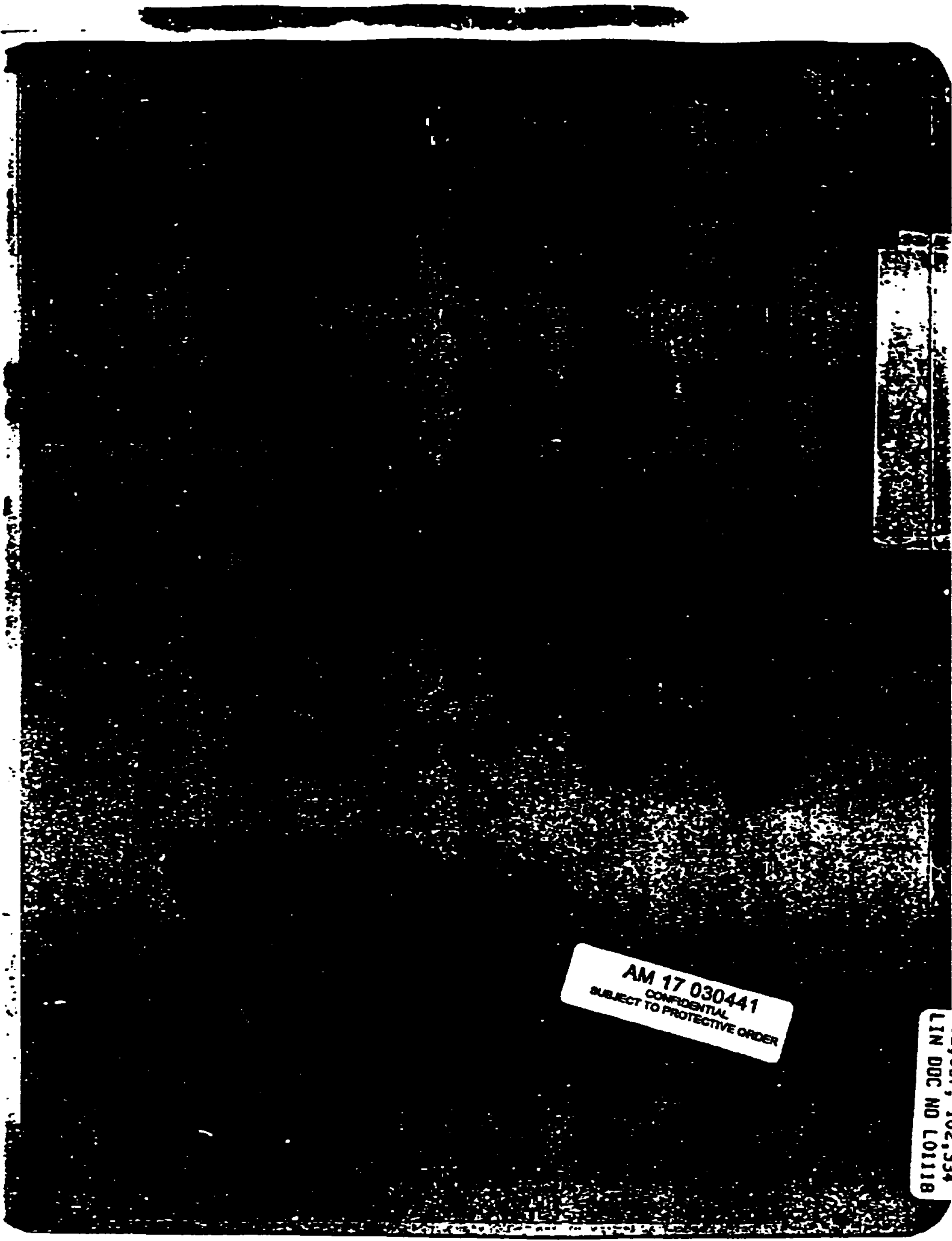
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FRITSCH v. LIN
INTER NOS 102,096
102,097, 102,334
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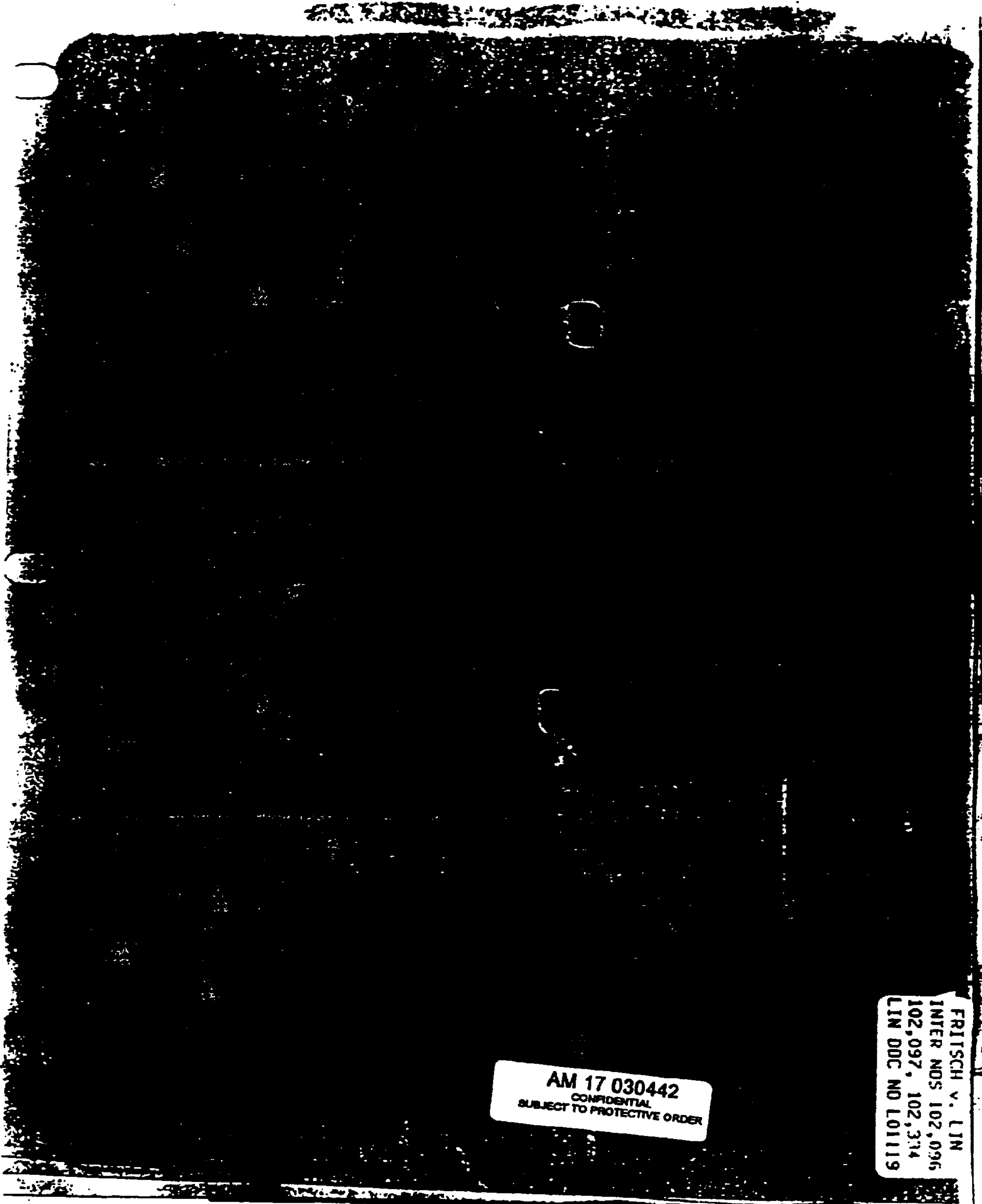
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FRITSCH v. LIN
INTER NOS 102,096
102,097, 102,334
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TITLE ____ Project No. ____ Book No. ____

From Page No. ____

Gel #1

1 Prestained low M.W. markers — 15λ
2 17λ of genes crude epo 1:10 — 40ng
3 Immppt. Cos cell mat'l — No Treatment
4 Immppt CHO cell mat'l — No treatment
5 17λ genes crude 1:10 + Endo. F — 40ng
6 Immppt cos cell mat'l + Endo F
7 Immppt. CHO cell mat'l + Endo.F
8 17λ genes crude 1:10 + neuraminidase — 40ng
9 Immppt cos cell mat'l + "
10 " CHO " + "

Gel #2

1 Prestained low m.w. markers — 15λ
2 13λ 1:10 crude epo + tachisorb (5λ Ab) — 30ng
3 " + " (10λ Ab) — 30ng
4 8.5 1:10 crude epo (suspend KH ... ON) — 20ng
5 5.5 1:10 " + B. subtilis extract — 20ng
6 8.5 1:10 " — 20ng
7 Kirin sample
8 yeast sample EPO 1 — 30λ
9 " EPO 2 — 30λ
10 " T6741 — 30λ

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Transfer to nitrocellulose for 6 hrs at 60 V at RT
Block ~2 hrs at RT w/ PBS + 10% horse serum.
Add mCA (P.A. #9) 13 ug/ml in PBS + 5% horse serum containing PMSF, trasylol, and azide per usual. Incubate ON
Wash 3x w/ PBS pH 7.6
Add biotinylated horse anti-mouse (1 drop/10ml PBS + 5% HS). Incubate 60 min
Wash 3x w/ PBS pH 7.6
Add ABC (2 drops each/10mls PBS) Incubate 1hr.
Wash 3x w/ PBS pH 7.6
Color development reagent as usual.

To Page No. ____

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 Project No.____ Book No.____ TITLE ____

From Page No.__

Results/Conclusions.

Gel #1

1) As previously seen the CHO cell untreated epo runs at a slightly larger molecular weight than Genes crude epo or COS cell expressed epo. After treatment w/ Endo F all 3 types of epo are shifted to a lower point. The COS and CHO material runs as a doublet - the upper band of each migrates w/ the spiked I-125 epo (see film overlay). This indicates that the larger MW of the CHO material is in fact due to carbohydrate. The doublet is frequently seen and may indicate some degradation at one end. Treatment w/ neuraminidase alone also apparently causes all 3 samples to migrate together and at the same apparent MW as spike I-125 epo.

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Gel #2

Using jacalin(?) to precipitate and then elute off seems to be a possible alternative to protein-A-Sepharose in that the background was very low. The recovery on most of the two samples was very poor - must look at this later.

Both of the tubes of genes crude epo appear to be OK - no degradation. The sample from Kirin seems to be running just slightly larger than genes epo. Need to [look at the] spike [with] an aliquot of this w/ I-125 epo to verify. The spike seems to migrate w/ Kirins sample so the effect may be due to salt or something.

In the yeast samples the background is so high that its impossible to determine.

Incubating genes crude epo w/ the B subtilis extract definitely causes a decrease in molecular weight indicating degradation. Will look at it more on the next western. i.e. can we inhibit the degradation by the addition of PMSF and Ca (then mark will know if its the two suspected proteases that are doing this.)

To Page ___

INTER NOS 102,096
102,097, 102,334
LIN DIC NO L01121

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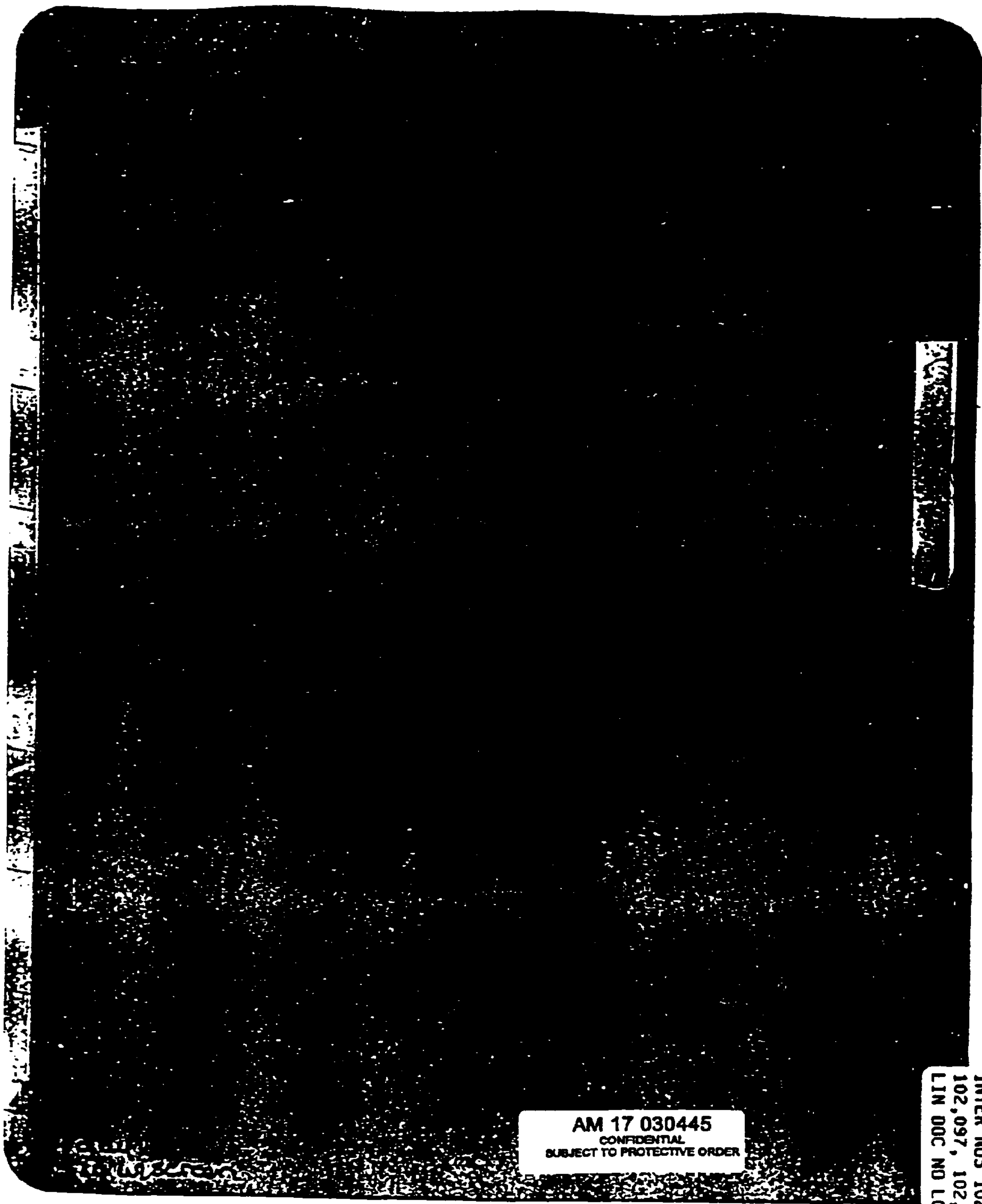

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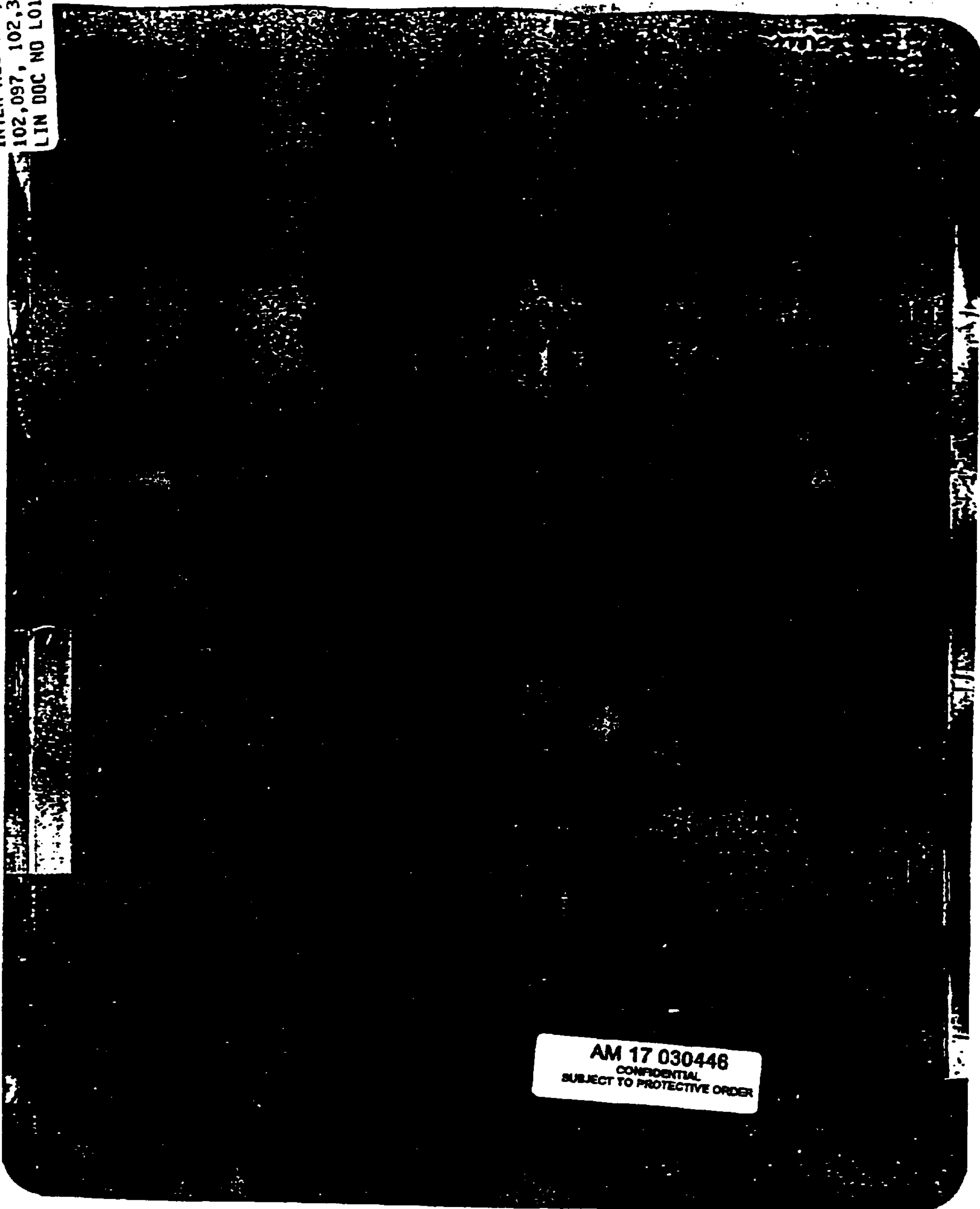
INTER NOS 102,096
102,097, 102,334
LIN DOC NO L01122

FRITSCH v. LIN
INTER NOS 102,096
102,097, 102,334
LIN DOC NO L01123



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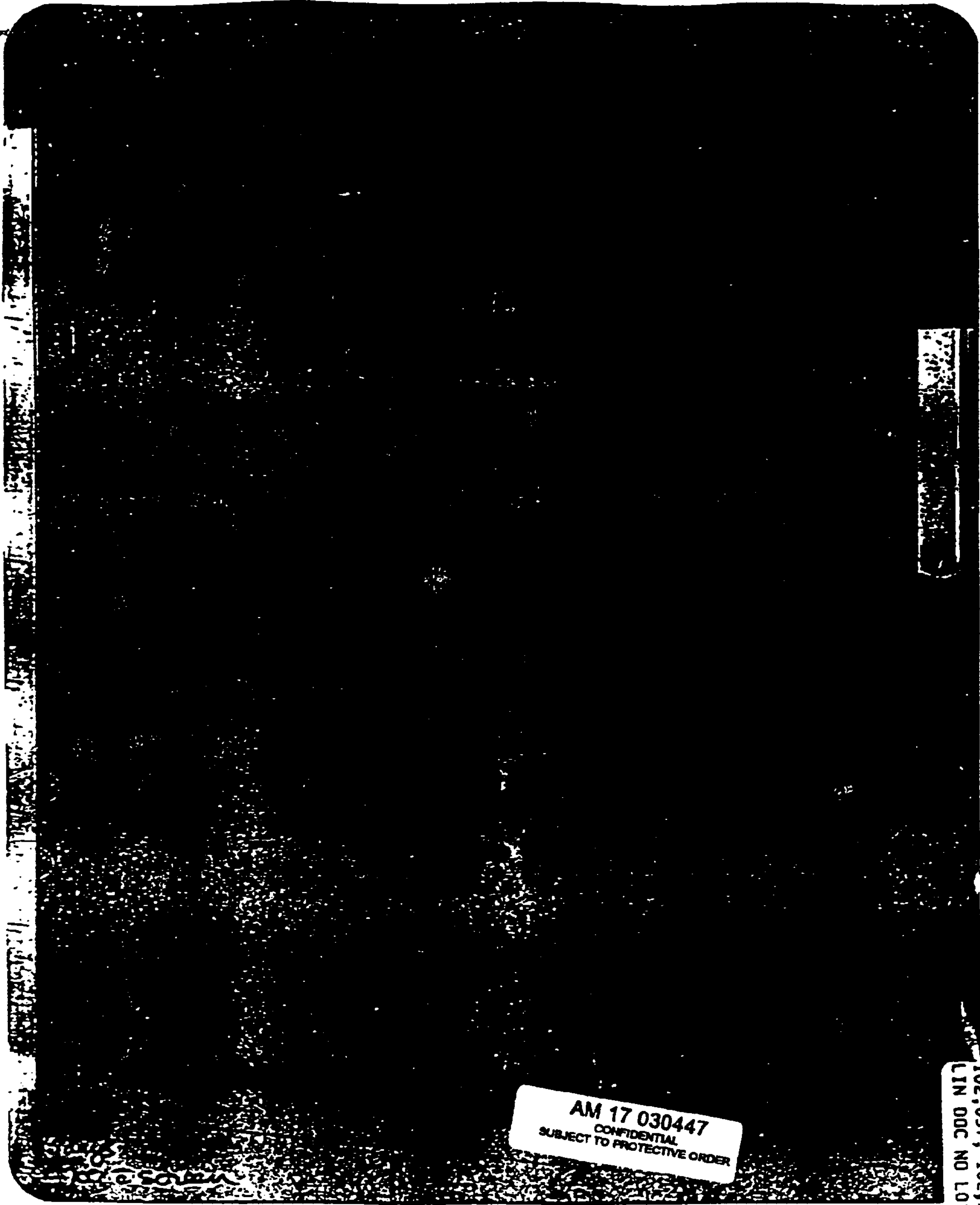
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INTER NOS 102,096
102,097, 102,334
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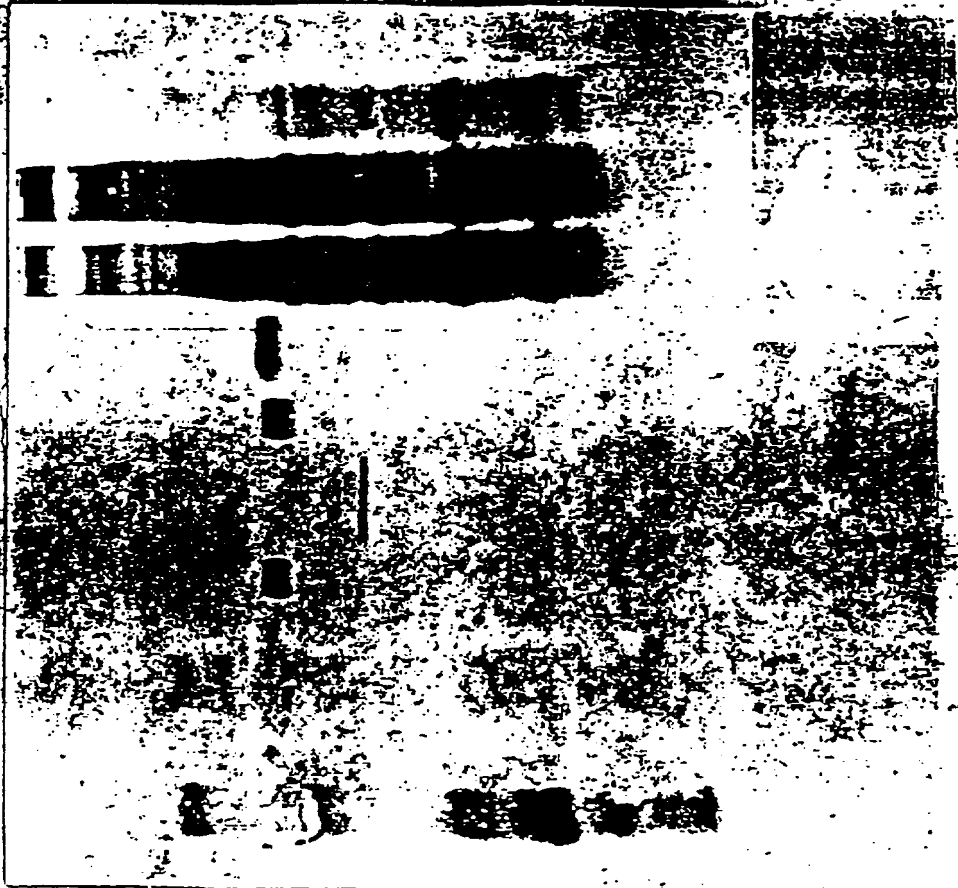

INTER NOS 102,096
102,097

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Project No.
Book No.
69

TITLE ① western of CHO material
② Coomassie of WGA fractions
③ Silver Stain of WGA fractions

From Page No.

7/10/84

Preparation for gel

① Immunoprecipitation of CHO samples
5λ trasylol / tube
PBS + 0.1% BSA so final volume is 400λ
Ab = 8C-204 #3089 + b3 - amt detailed below
spike all samples w/ 10λ I-125 epo (cuss) 27,304 cpm
O.N. at 4°
(8λAb) ~ 75 - 100λ (10λAb) protein A-sepharose 1:5
wash as usual, count
extract w/ 80λ 3x LSB

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sample		Ab	cpm	%	pellet	sup	%	Approx amt on gel
					60	60	—	
1 pooled sample	5u = 71λ	PI	72	0.5%				
2 4d F 10d	2.5u = 35.5λ	8λ	23132 / 6624	27.4% / 86.6%	476	1519	76.1%	
3 B11 hEPO	5u = 71λ	8λ	6500	85.0%	1622	4639	74.1%	
4 70 u/ml	10u = 143λ	8λ	2199	28.8%	453	1564	77.5%	
5 Genescrude	400ng = 17.6λ	8λ	2442	31.9%	492	1819	78.7%	10
6 1:10	200ng = 8.3λ	8λ	3134	41.0%	452	2468	84.5%	6.9
7	200ng = 8.3λ	PI	153	2%	96	88		—
8 CHO-mEPO	5.45u = 175λ	80λ	6962	54.5%	1423	4794	76.5%	
9 serum free 1 μm mix 312u/ml	10.9u = 35λ	10λ	6507	85.1%	1229	4836	79.7%	
10 mock		8λ	7649	100%				

* lost all but this and when tube popped open and fell in boiling water

② Protein concentrations on
A. Ivi Weissmans' dissected mouses ascites fluid

	A280	A260	A235	A230	230/260	235/280
1:1000	.104	.121	.249	.330	51.51 mg/ml	57.77 mg/ml

B. 2 fractions from the urine purification - after WG column
CBB - 7978.4 / A280 [per Tom 5/22/84 A280 = .641/ml volume = 350λ
DCB - 32234 / A280 [5/31/84 A280 = .828 A / ml volume = 1.35 mls

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by	

FRITSCH v. LIN
INTER NOS 102,09
102,097, 102,334
LIN DOC NO L0112

 Project No. ______ Book No. ______ TITLE ______

From Page No. ___

	A250	A260	A235	A230	230/260	235/280
CBB	.609	.450	1.535	2.35	.413	.488 mg/ml
DCB	.229	.546	2.241	2.90	.522 mg	.583 mg/ml

my calculation of protein using the 235/280 formula gives about 70-75% as much protein as the A280 number Tom gave me. For our consistencies sake I will use my number as the amt.

CBB = .75 x .488 mg/ml = 366 ug total protein
7978 U/A280 x .641 A280/ml = 5114 U/ml x .75 mls = 3835 U total
3835 U total / 366 mg = 10,478 U/mg   10475 U/mg / 70,000 [U/mg of pure epo] = 15% pure
15% x 366 ug = [54.9 ug epo]

DCB - 1.35 x .583 mg/ml = 787 total protein
3223 U/A280 x .828 A280/ml = 2669 U/ml x 1.35 mls = 3603 U total
3603 U total / 787 mg = 4,582 U/mg   4582 / 70,000 = 6.55% pure
6.55% x 787 ug = [51.5 ug epo]

Gel - poured two 12.5% acrylamide gels. Ran at 35 v O.N.

#1
1 prestained mw markers - 15λ
2 pooled material + PI
3 h EPO + Ab - 25 u start
4 " + Ab 50 u start
5 " + Ab 100 u start
6 genes' crude 1:10 + Ab 40ng "
7 " 20ng "
8 " + PI
9 serum free - mepo + Ab 5.45 u std
10 " + Ab 10.9 u "

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#2

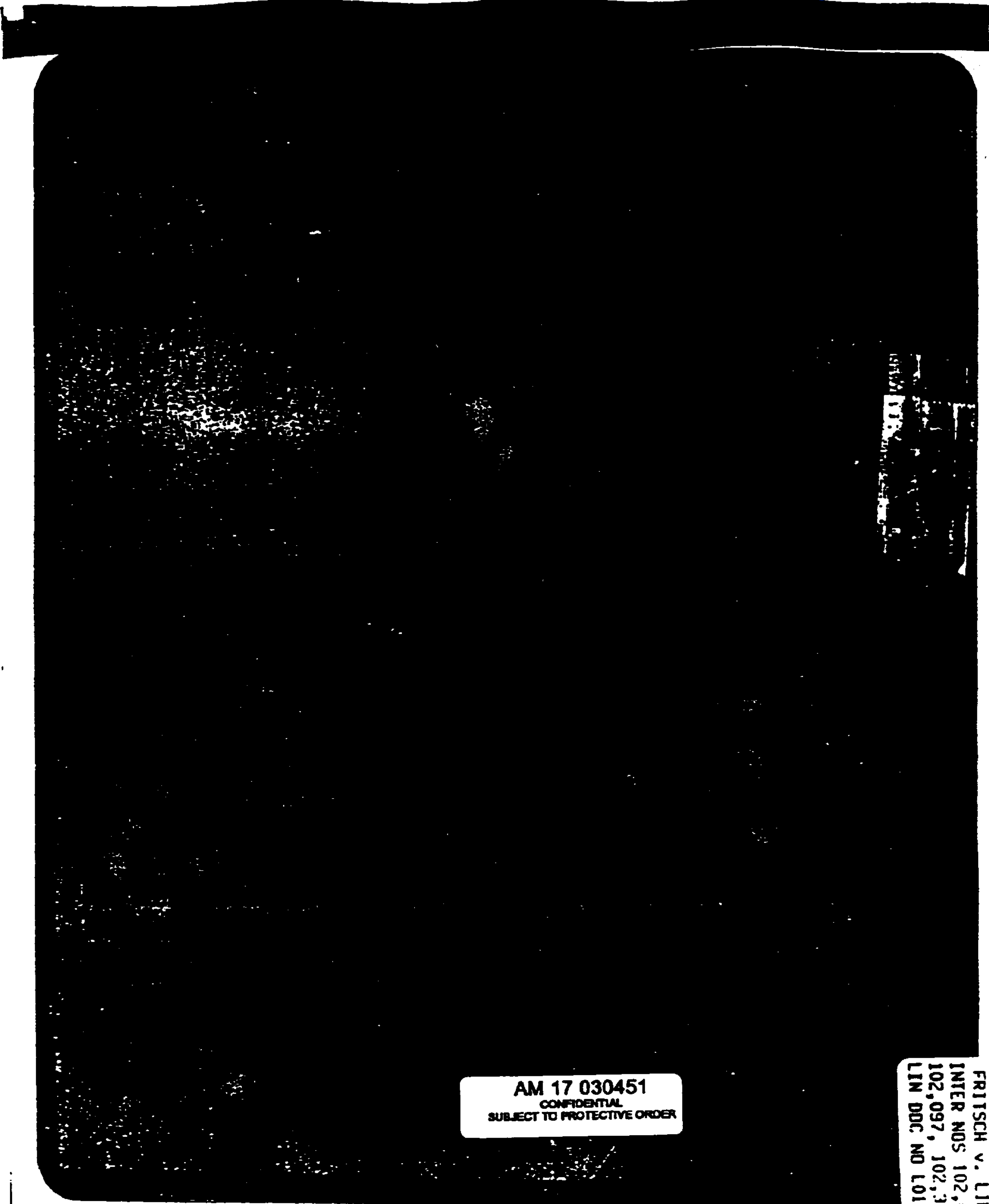
for coomassie stain	for silver stain
1 mw - mouse - ascites 300ug - 55λ 1:10	6 CBB - .5 ug [~ 10% pure ~ 50ng epo] - 1λ
*2 " " 150ug - 27.5λ 1:10	7 " 1.0 ug " 100ng epo - 2λ
3 CBB - 48.8 ug [~ 10% pure ~ 5 ug epo] 100λ	8 DCB - 1.0 ug [~ 5% pure ~ 50ng epo] - 1.7λ
4 DCB - 72.9 ug [~ 5% pure ~ 4 ug epo] 125λ	9 " 2.0 ug [" 100ng epo - 3.4λ
*5 prestained markers - 15λ	10 standards - 10ng each -
cut	

* spiked with 10,953 cpm/lane

To Page No. ____

Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by	

FRITSCH v. LIN
INTER NOS 102.096
102,097, 102,334
LIN DOC NO L01127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FRITSCH v. LIN
INTER NOS 102,096
102,097, 102,334
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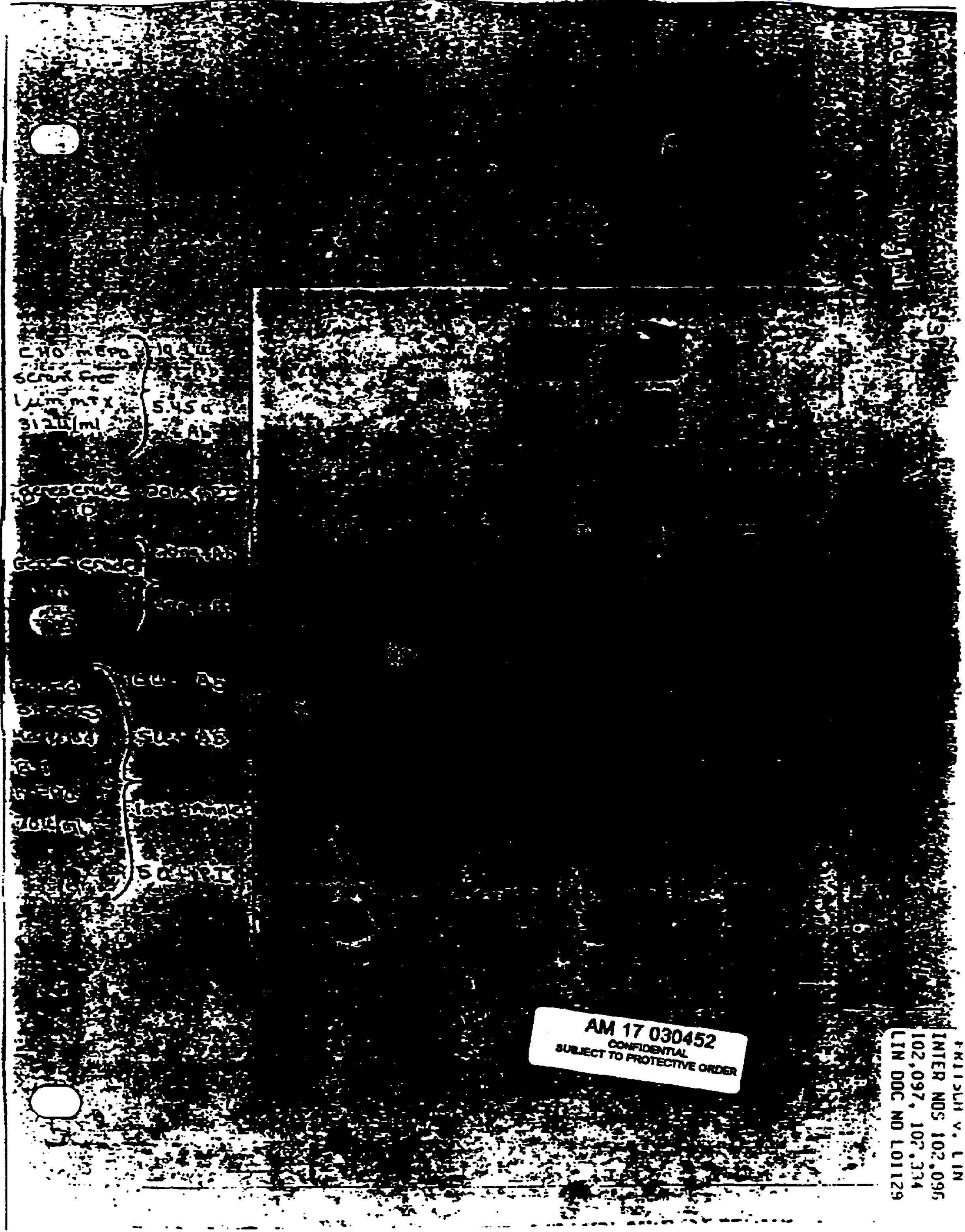

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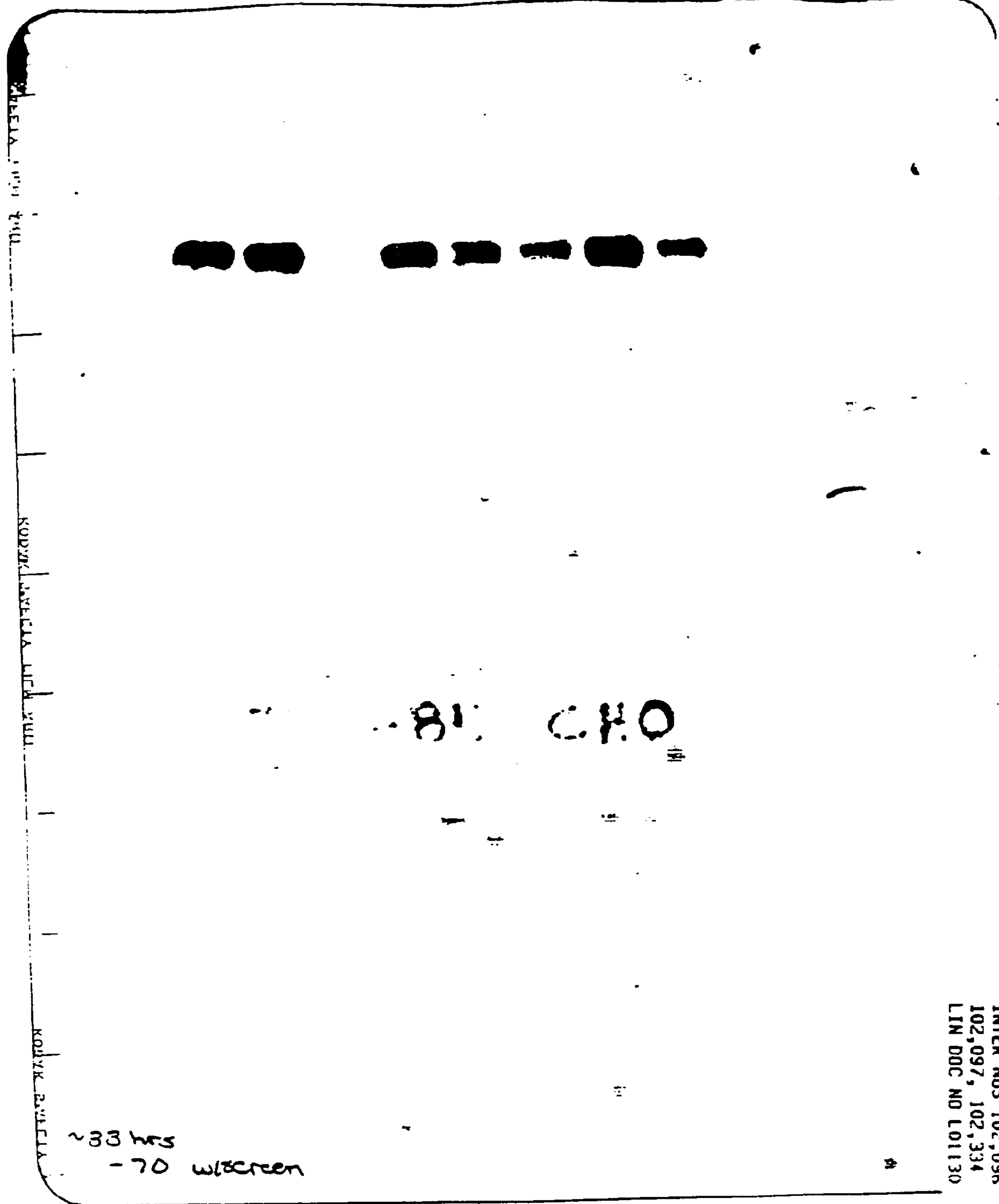

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-8 CHO

7/16/84 3 days
-70 w/screen

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FRITSCH v. LIN
INTER NOS 102,096
102,097, 102,134
LIN DOC NO 101131

Project No.
Book No.

TITLE

From Page No.

Gel # 1 - transferred to nitrocellulose: 60v for 5½hrs
Blocked with 10% horse serum for 1 hr
Incubate with mcA overnight - 13ug/ml of protein A sepharose + 9 in 5% horse serum; 100 units/ml trasylol; .5mm PMSF; and 0.025% Na azide
Wash 3X w/ PBS- pH 7.6
Add biotinylated horse anti-mouse in 5% horse serum 1 hr at RT
Wash 3X w/ PBS- pH 7.6
Add ABC 60 min at RT
Wash 3X w/ PBS- pH 7.6
Add color development reagent

Gel #2 - cut in half - stain lanes 1-5 w/ coomassie for ~2 hrs
Destain overnight. Soak in 3% glycerol Dry Expose to film.
lanes 6-10 silver stain as follows:

1. Fix overnight in { 200 mls methanol, 48 mls HOAC, 152 ml H_2O } (usually this is done for 30 min - but I couldn't get to it till the next day)
2. Wash 3X in 300 mls of 10% ETOH; 5% HOAc
3. Soak gel in 400 mls of 5ug/ml DTT for 30 minutes
4. Soak in 200 mls of 12mm $AgNO_3$. Expose to fluorescent light for first five minutes - then allow to sit another 40 minutes
5. Wash w/ ~400 mls of H_2O - 2X for ~2 min each
6. Prepare fresh developer { .28m Na_2CO_3 (29.7g/l), .5mls of 37% formalin/l }

1x 1 min
1x ~3 min
1x 5 min or until reaches desired intensity

7. Stop with 400 mls of 1% HOAc (~5 min)
8. Wash 2X w/ 500 mls H_2O

Soak in 3% glycerol
Dry
Expose to film

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FRITSCH v. LIN
INTER NOS 102,096
102,097, 102,334
LIN DOC NO L01132

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by	

72

Project No.
Book No. TITLE

From Page No.

Results-

Gel #1 western -

CHO-m epo in serum free media at 1μm MTX - we had never looked at this material on a western before - so we wanted to check that the epo was the usual size in high MTX and in the serum free media (we've worried about limiting carbohydrate or other factors). The material ran as usual - just slightly larger than Genes crude epo.

CHO - h epo - this had never been looked at by a western before (only h-epo expressed in COS cells) This material runs like m-epo expressed in CHO - just slightly larger than genes crude epo. The size of m-epo and h-epo appear to be the same in CHO indicating the slightly larger size is probably due to CHO-specific carbohydrate structures.

The western was dried and exposed on film - since all samples were spiked with our I-125 epo, upon overlaying the film to the western a most unusual result was observed: our I-125 epo appeared to migrate with the CHO expressed material and slightly larger than genes crude epo. we will repeat this - and run genes I-125 next to it. The significance of this observation would be that naturally occuring epo migrates slightly differently - depending on its carbohydrate composition.

Gel #2

(A) coomassie stained material - Both wheat germ fractions had a strong band which co-migrated (one migrated slightly above) with Genes I-125 epo. In both cases there was enough epo present so the material could be used by Ab, Inc to boost rabbits. As for the ascites from the mouse Ira Weissman dissected - there is a band visible which co-migrates with I-125 epo from genes.

(B) In the silver stained gel I made the following observations: the bands which co-migrate with Genes I-125 epo (one migrates slightly above) just like in the coomassie gel) came up very quickly and with little background. I allowed the gel to continue staining so I could pick-up background - In the case of the silver stain - the presumed epo bands seemed to be at least 50% of the staining material. Either epo silver stains very well or coomassie stains very poorly. Unfortunately I dried the gel down on paper so the bands aren't very distinct now.

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To Page No.

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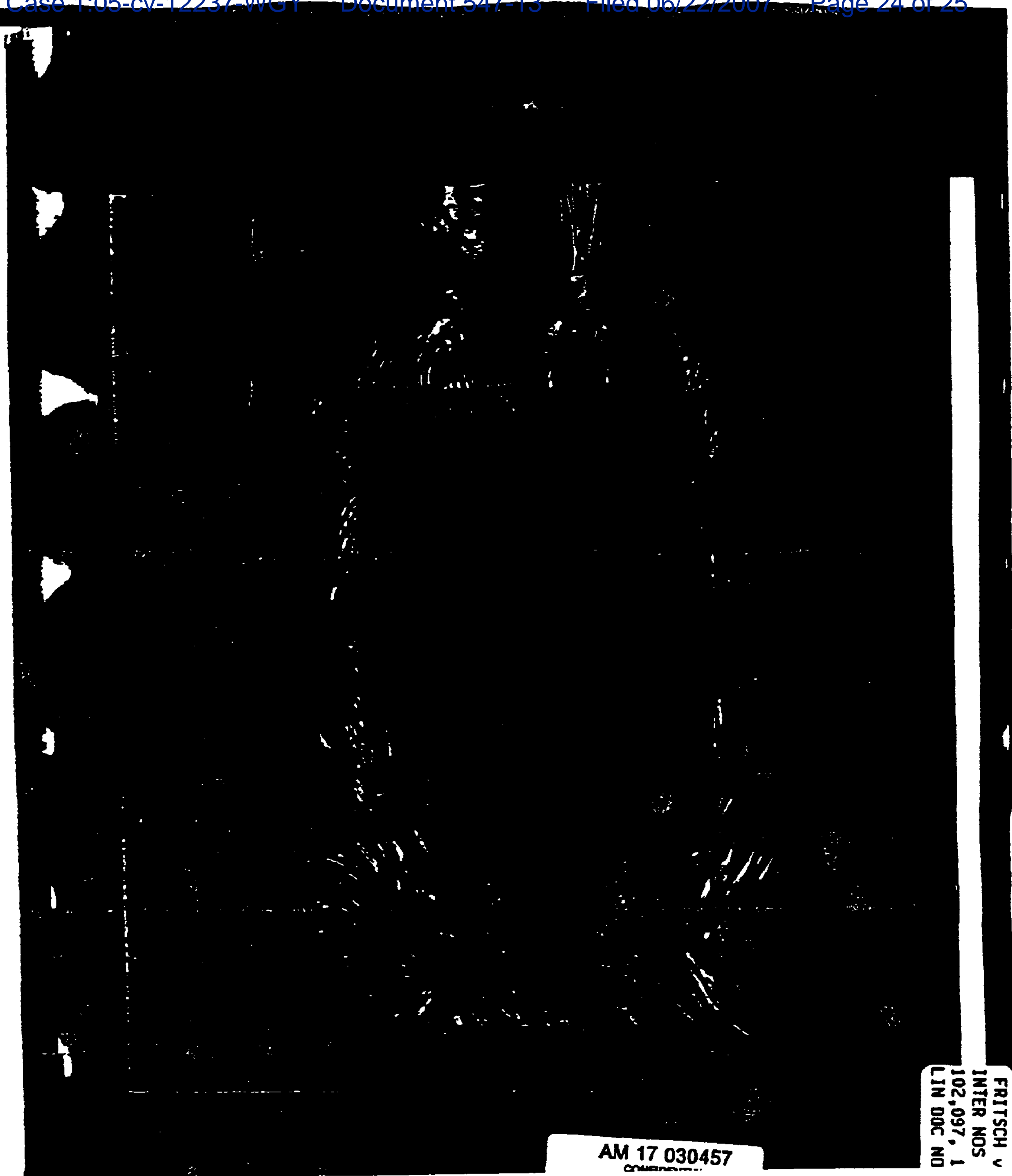
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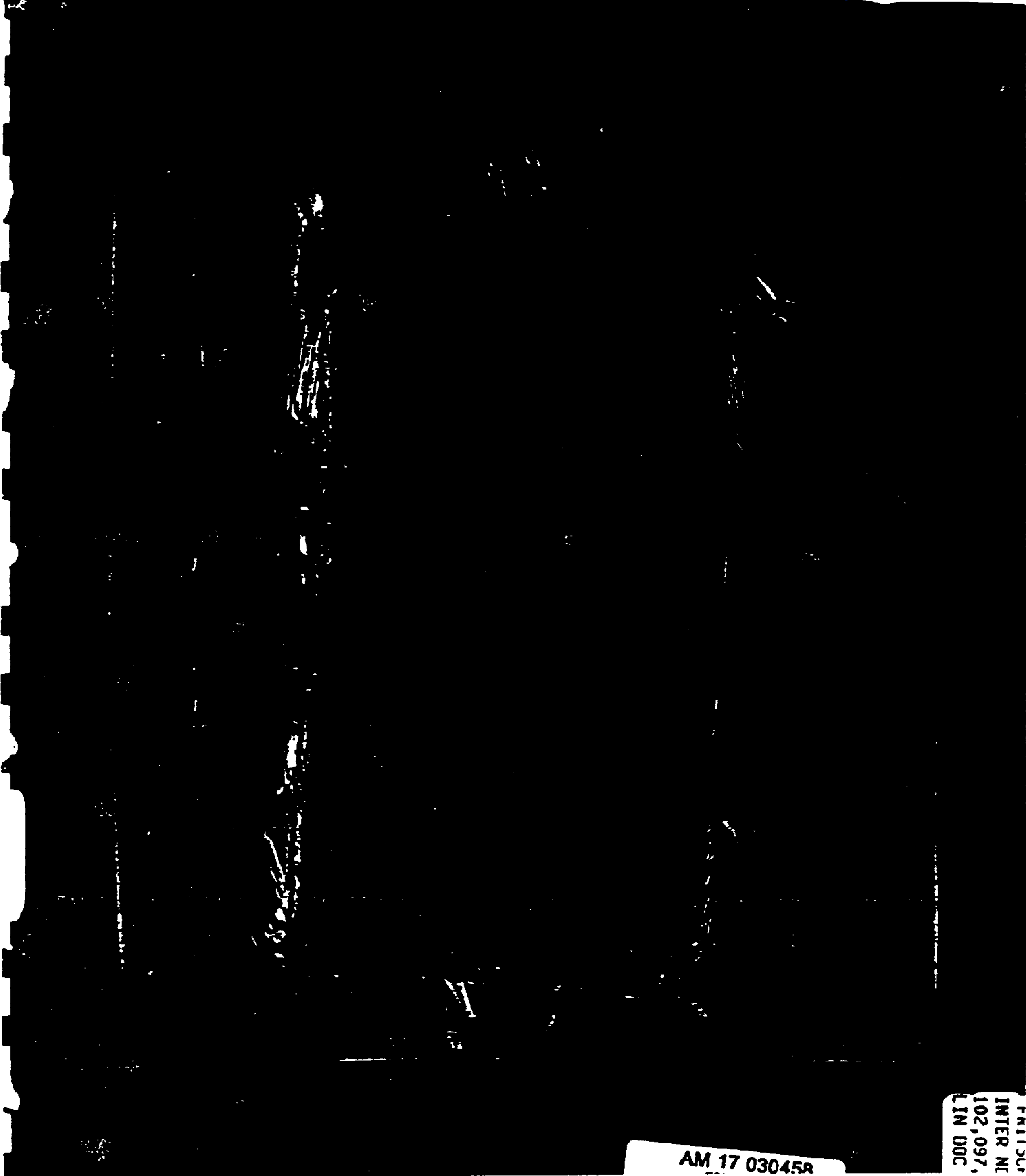
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