



CYP IMMUNOINHIBIT KIT

PRODUCT# Hu-A011A, Hu-A011B

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1. **Description**

The *CYP ImmunoInhibit Kit* from CYP450-GP is designed for performing P450 enzyme reaction phenotyping studies in model metabolic systems, especially human liver microsomes (HLMx). The inhibitory polyclonal and monoclonal (mAb) antibodies to P450 enzymes contained in this kit are powerful tools for assessing whether a particular P450 catalyzes the oxidative metabolism of a given therapeutic agent/ lead compound. These P450 antibodies, upon binding to their cognate enzyme, markedly decrease (up to 90%) that enzyme's metabolic activity. These inhibitory IgGs are effective not only against P450s found in native HLMx but also give marked metabolic inhibition of purified, reconstituted P450 enzymes, heterologously-expressed P450s (e.g., Supersomes™) and permeabilized human hepatocytes [1-4]. For example, optimal amounts of anti-human CYP3A4 IgG elicit 90%, 98% and 90% inhibition of exemplary CYP3A4 substrate metabolism by HLMx, purified human CYP3A4 and MetMax™ hepatocytes, respectively. To achieve this extent of inhibition with *CYP ImmunoInhibit Kit* antibodies, two criteria must be satisfied:

- A)** adequate amounts of IgG must be included in the incubation mixture;
- B)** the drug-metabolizing reaction being tested for inhibition must be catalyzed predominantly by the P450 to which the antibody has been produced. In those instances where the extent of immunoinhibition is substantially less (e.g, 30-50%), other P450 enzymes are likely partaking in the reaction.

CYP ImmunoInhibit Kit antibodies can be employed in substrate metabolism studies as well as in substrate depletion/stability experiments with HLMx when labeled substrate or authentic metabolite standards are not available. The results obtained are easily interpreted, and do not require extrapolation (e.g., ISEF, RAF) to assess the contribution of a given microsomal P450 enzyme to lead compound metabolism.



2. Components

The polyclonal antibodies comprising the *CYP ImmunoInhibit Kit* have been developed in rabbits using purified human native or recombinant CYP1A2, CYP2C19, CYP2D6 or CYP3A4 as immunogens. mAb-containing ascites fluid was produced in mice using hybridomas derived from animals immunized with recombinant human CYP2C8 and CYP2C9. Whole IgG fractions were purified from antisera or ascites fluid using caprylic acid/ammonium sulfate fractionation. Preimmune (control) IgG was derived from rabbit serum prior to immunization. The individual polyclonal and mAb IgGs are provided as powders after lyophilization from 100 mM potassium phosphate buffer (pH 7.4), 150 mM KCl, and 2.5 μ M thimerosal.

Note: The *CYP ImmunoInhibit Kit* does not include inhibitory antibodies to human CYP2B6. Instead, the kit contains a ready-to-use aliquot of 2-phenyl-2-(1-piperidinyl)propane or PPP, a potent and specific human CYP2B6 chemical inhibitor [5].

3. Storage and Reconstitution of Antibodies

Store kit containing the lyophilized IgGs at -20°C . Reconstitute by adding 100 μ l (for 1 mg kit) or 200 μ l (for 2 mg kit) of 100 mM potassium phosphate buffer, pH 7.4 (or another suitable reaction buffer) to each vial of polyclonal IgG, and mix vial gently until powder dissolves, giving a final antibody concentration of 10 mg IgG/ml. These volumes should be adjusted to 250 - 500 μ l for preimmune IgG to achieve the same antibody concentration. CYP2C8 and CYP2C9 mAb IgGs are reconstituted in the same manner but the final antibody concentration will be 1 mg IgG/ml. After reconstitution, the antibody solutions should be stored at -20°C , taking care to avoid extensive freeze/thaw cycles.



CYP IMMUNOINHIBIT KIT COMPONENTS

Antibody	Kit Amount	Model Substrate/ Reaction	Extent of Inhibition
Anti-CYP1A2 (Hu-A010)	1-2 mg [Yellow Cap]	Phenacetin <i>O</i> - Deethylase	>80% at 3.0 mg IgG/mg HLMx
Anti-CYP2C8 (Hu-A004M)	100-200 µg [Blue Cap]	Paclitaxel 6α- Hydroxylase	>85% at 0.04 mg IgG/mg HLMx
Anti-CYP2C9 (Hu-A003M)	100-200 µg [Pink Cap]	Diclofenac 4'-Hydroxylase	>80% at 0.075 mg IgG/mg HLMx
Anti-CYP2C19 (Hu-A008)	1-2 mg [Orange Cap]	<i>S</i> -mephenytoin 4'-Hydroxylase	>90% at 1.0 mg IgG/mg HLMx
Anti-CYP2D6 (Hu-A009)	1-2 mg [Green Cap]	Dextromethorphan <i>O</i> -Demethylase	>80% at 2.0 mg IgG/mg HLMx
Anti-CYP3A4 (Hu-A005)	1-2 mg [Red Cap]	Nifedipine Oxidation	>85% at 2.0 mg IgG/mg HLMx
Preimmune (Hu-A000)	2-4 mg [White Cap]	n/a	n/a

Inhibitory characteristics of the antibodies listed are given in more detail in their individual product descriptions (see <http://cyp450-gp.com/about/p450-antibodies>)

SPECIFICITY OF CYP IMMUNOINHIBIT KIT ANTIBODIES

P450 ENZYME	ANTIBODY					
	Anti- CYP1A2	Anti- CYP2C8	Anti- CYP2C9	Anti- CYP2C19	Anti- CYP2D6	Anti- CYP3A4
CYP1A2	+++	0	0	0	0	0
CYP2A6	0	0	0	0	0	0
CYP2C8	0	+++	0	0	0	0
CYP2C9	0	0	+++	0	0	0
CYP2C19	0	0	0	+++	0	0
CYP2D6	0	0	0	0	+++	0
CYP3A4	0	0	0	0	0	+++
CYP3A5	0	0	0	0	0	++
CYP4A11	0	0	0	0	0	0

Antibody specificity was determined by Western blotting and/or ELISA (<http://cyp450-gp.com/about/p450-antibodies>)



4. Antibody Inhibition Assays

The first immunoinhibition assay described below employs *CYP ImmunoInhibit Kit* antibodies to identify the P450 enzyme(s) catalyzing a drug-metabolizing reaction in HLMx. This is a typical P450 reaction phenotyping study. The capacity of P450 antibodies to inhibit microsomal oxidation of a given test compound (in this case, *O*-demethylation of dextromethorphan; DXM) is being assessed. Upon finding that only anti-CYP2D6 gives extensive inhibition of DXM metabolism, an immunotitration assay can then be performed to reveal the overall extent of CYP2D6 involvement in this reaction. The IgG:microsomal protein ratio giving optimal inhibition of dextromethorphan formation can subsequently be employed with other distinct HLMx.

P450 REACTION PHENOTYPING ASSAY

I. Reagents Needed

- A) 100 mM potassium phosphate buffer, pH 7.4, at room temperature
 B) 2.5 mM dextromethorphan (DXM) - Prepare by dissolving 4.6 mg DXM hydrobromide monohydrate in 5 ml of H₂O
 C) 10 mM NADPH - Prepare fresh by dissolving 5.6 mg in 0.6 ml Reagent A
 D) Acetonitrile
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II. Assay Format

<u>Tube #</u>	<u>HLMx</u>	<u>Antibody</u>	<u>NADPH</u>	<u>Other Rx Components</u>
1	2.5 μ l	10 μ l A	--	+
2	2.5 μ l	10 μ l A	--	+
3	2.5 μ l	10 μ l A	--	+
4	2.5 μ l	10 μ l A	+	+
5	2.5 μ l	10 μ l A	+	+
6	2.5 μ l	10 μ l A	+	+
7	2.5 μ l	10 μ l B	+	+
8	2.5 μ l	10 μ l B	+	+
9	2.5 μ l	10 μ l B	+	+
10♦	2.5 μ l	10 μ l A + 2 μ l C	+	+
11	2.5 μ l	10 μ l A + 2 μ l C	+	+
12	2.5 μ l	10 μ l A + 2 μ l C	+	+
13	2.5 μ l	10 μ l A + 4 μ l D	+	+



14	2.5 μ l	10 μ l A + 4 μ l D	+	+
15	2.5 μ l	10 μ l A + 4 μ l D	+	+
16	2.5 μ l	10 μ l E	+	+
17	2.5 μ l	10 μ l E	+	+
18	2.5 μ l	10 μ l E	+	+
19	2.5 μ l	10 μ l F	+	+
20	2.5 μ l	10 μ l F	+	+
21	2.5 μ l	10 μ l F	+	+
22	2.5 μ l	10 μ l G	+	+
23	2.5 μ l	10 μ l G	+	+
24	2.5 μ l	10 μ l G	+	+
<u>[IF INCLUDED]</u>				
25	2.5 μ l	5 μ l PPP [•]	+	+
26	2.5 μ l	5 μ l PPP	+	+
27	2.5 μ l	5 μ l PPP	+	+
28-30	2.5 μ l	5 μ l MeOH [•]	+	+

HLMx (pool of 50) = 20 mg protein/ml; 2.5 μ l = 50 μ g protein

A: Preimmune IgG, 10 mg IgG/ml; **B:** Anti-CYP1A2 IgG, 10 mg IgG/ml
C: Anti-CYP2C8 mAb IgG, 1 mg IgG/ml; **D:** Anti-CYP2C9 mAb IgG, 1 mg IgG/ml
E: Anti-CYP2C19 IgG, 10 mg IgG/ml; **F:** Anti-CYP2D6 IgG, 10 mg IgG/ml
G: Anti-CYP3A4 IgG, 10 mg IgG/ml

IgG:HLMx Ratio = 2.0 mg IgG/mg protein

mAb IgG:HLMx Ratio = 0.04-0.08 mg IgG/mg protein

◆ Additional preimmune IgG is added to tubes 10-15 in order to keep the antibody concentration constant among assay tubes, thereby avoiding non-specific immunoglobulin effects.

● The glass vial marked **PPP** included in the kit contains 40 nmol PPP in dried form. Reconstitute the solid by adding 50 μ l MeOH (or another appropriate solvent) to the vial, and mix well, giving a 800 μ M PPP solution. Addition of 5 μ l of this solution as shown above (see tubes 25-27) will result in a final concentration of 16 μ M PPP. Controls for this compound should contain 5 μ l of solvent alone (see tubes 28-30).



III. Procedure

1. Add 50 μl of assay buffer (reagent A) to 1.5 ml Eppendorf-type microfuge tubes, keeping in mind that the final assay volume is 250 μl .

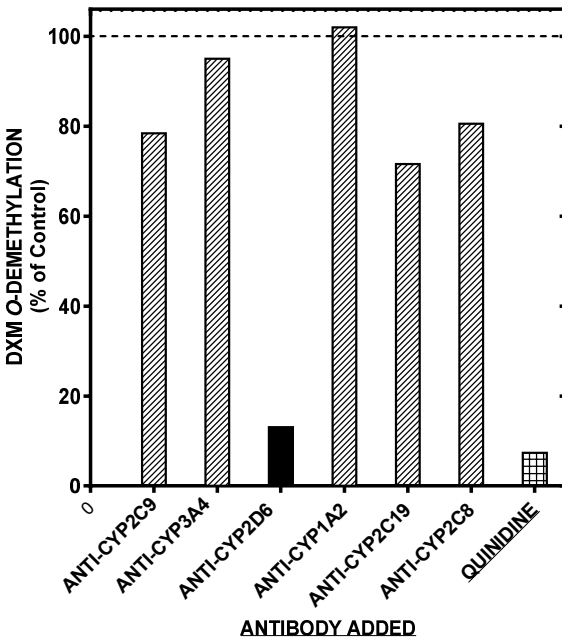
Note: This assay can also be performed in 96-well plates (200 μl volume) but with a proportional reduction of reagent amounts.

2. Add the above-described amounts of immune-specific and/or preimmune IgG to the tubes. Then, add liver microsomes (equivalent to 50 μg protein). A minimum of 24 tubes is needed to test each antibody in triplicate with the inclusion of 3 tubes without the cofactor NADPH. An additional 6 tubes are needed if PPP inhibition is also being tested.

3. Incubate tubes **for 3 min @ 37°C** with shaking, then let stand for **10 min at room temp.** Finally, place tubes back onto ice.

4. Add remaining components to the tubes, taking into account the prior addition of 50 μl assay buffer. These include the assay buffer (reagent A) and substrate (reagent B, 100 μM final concentration).

5. Start reactions with 25 μl of 10 mM NADPH (reagent C), and incubate for 15 min at 37°C. Omit NADPH from negative controls (tubes 1-3).





6. Terminate reactions with 100 μ l of acetonitrile (reagent D), and analyze for the *O*-demethylated metabolite dextrorphan [6-8].

7. Plot % of control (or % inhibition), as determined from metabolism rates obtained in the presence of control IgG versus rates obtained in presence of immune-specific IgG.

As shown in the figure above, only CYP2D6 antibodies elicited marked (87%) inhibition of microsomal DXM *O*-demethylation while the other five *CYP ImmunoInhibit Kit* antibodies tested had little effect on this reaction. Note that the CYP2D6 chemical inhibitor quinidine was included in this antibody-based phenotyping analysis for comparative purposes.

P450 METABOLISM IMMUNOTITRATION ASSAY

The second assay presented here describes construction of an antibody titration curve, which should be performed subsequent to P450 reaction phenotyping. An immunotitration curve identifies the ratio of antibody:microsomal protein required to give optimal (> 80%) inhibition of substrate metabolism, in this case, DXM *O*-demethylation with anti-CYP2D6. Importantly, antibody titration curves can often reveal whether a single or multiple P450s catalyze a given drug-metabolizing reaction. However, the phenotyping study given above is often sufficient for P450 catalyst identification purposes, especially when combined with complementary methodology (e.g, metabolic screening with individual recombinant P450 enzymes).

I. Reagents Needed

- A) 100 mM potassium phosphate buffer, pH 7.4, at room temperature
- B) 2.5 mM dextromethorphan (DXM) - prepared as described above
- C) 10 mM NADPH - prepared as described above
- D) Acetonitrile

II. Assay Format

<u>Tube</u>	<u>HLMx</u>	<u>Antibody</u>	<u>NADPH</u>	<u>Other Rx Components</u>
1	2.5 μ l	15 μ l A	--	+
2	2.5 μ l	15 μ l A	--	+
3	2.5 μ l	15 μ l A	--	+
4	2.5 μ l	15 μ l A	+	+



5	2.5 μ l	15 μ l A	+	+
6	2.5 μ l	15 μ l A	+	+
7	2.5 μ l	13 μ l A + 2 μ l B	+	+
8	2.5 μ l	13 μ l A + 2 μ l B	+	+
9	2.5 μ l	13 μ l A + 2 μ l B	+	+
10	2.5 μ l	10 μ l A + 5 μ l B	+	+
11	2.5 μ l	10 μ l A + 5 μ l B	+	+
12	2.5 μ l	10 μ l A + 5 μ l B	+	+
13	2.5 μ l	5 μ l A + 10 μ l B	+	+
14	2.5 μ l	5 μ l A + 10 μ l B	+	+
15	2.5 μ l	5 μ l A + 10 μ l B	+	+
16	2.5 μ l	15 μ l B	+	+
17	2.5 μ l	15 μ l B	+	+
18	2.5 μ l	15 μ l B	+	+

HLMx (pool of 50) = 20 mg protein/ml; 2.5 μ l = 50 μ g protein

A = Preimmune IgG, 10 mg IgG/ml

B = Anti-CYP2D6 IgG, 10 mg IgG/ml

IgG:HLMx Ratios = 0, 0.4, 1.0, 2.0 & 3.0 mg IgG/mg protein

Note: This assay incorporates a constant amount of IgG (anti-CYP2D6 IgG plus preimmune IgG = 0.15 mg) in each assay tube to avoid any non-specific immunoglobulin effects.

Note: This assay requires 1.0 mg of anti-CYP2D6 IgG to perform as outlined, or 0.7 mg of this immune-specific antibody when done using replicate tubes. If required, additional amounts of CYP2D6 antibody or any other immune-specific antibody included in the *CYP ImmunoInhibit Kit* can be ordered as a separate product.

III. PROCEDURE

1. Add 50 μ l of assay buffer (reagent A) to 1.5 ml Eppendorf-type microfuge tubes, keeping in mind that the final assay volume is **250 μ l**.

Note: This assay can also be performed in 96-well plates (200 μ l volume) but with a proportional reduction of reagent amounts.

2. Add the above-described amounts of immune-specific and/or preimmune IgG to the tubes. Then, add liver microsomes (equivalent to



50 μ g protein). A minimum of 18 tubes is needed to test each antibody: microsomal protein ratio.

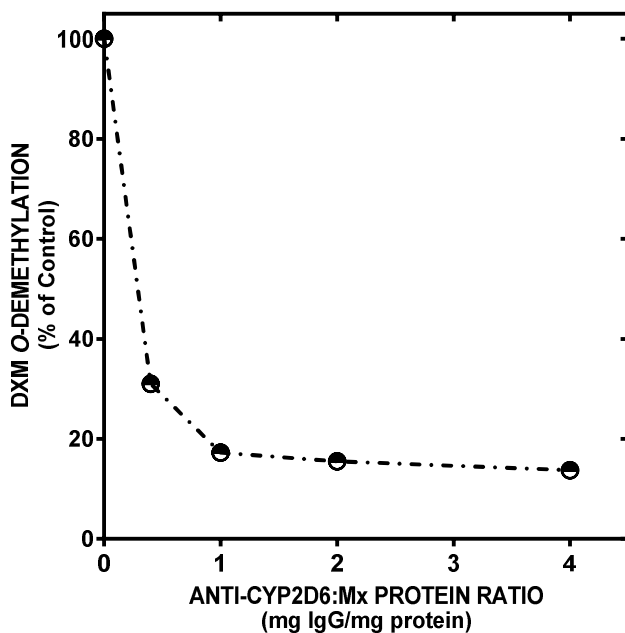
3. Incubate tubes **for 3 min @ 37°C** with shaking, then let stand for **10 min at room temp.** Finally, place tubes back onto ice.

4. Add the remaining components to the tubes, taking into account the prior addition of 50 μ l assay buffer. These include the additional assay buffer (reagent A) and substrate (reagent B, 100 μ M final concentration).

5. Start reactions with 25 μ l of 10 mM NADPH (reagent C), and incubate for 15 min at 37°C. Omit NADPH from negative controls (tubes 1-3).

6. Terminate reactions with 100 μ l of acetonitrile (reagent D), and analyze for the *O*-demethylated metabolite dextrorphan [6-8].

7. Plot % of control (% activity remaining) versus antibody:microsomal protein ratio, as determined from metabolism rates obtained in the presence of control IgG versus those obtained in presence of increasing amounts of anti-CYP2D6 IgG. The results shown below indicate that CYP2D6 is the major, if not exclusive, catalyst of DXM *O*-demethylation in human liver.





5. Troubleshooting

SYMPTOM	CAUSE
<i>CYP ImmunoInhibit Kit</i> antibodies fail to elicit inhibition	a) Antibody: microsomal protein ratio used is suboptimal; b) Buffer volume used to preincubate HLMx with IgGs is too large or preincubation time is too short; c) An enzyme other than a major drug-metabolizing P450s is the catalyst (e.g., CYP2B6 or CYP2E1).
Partial inhibition of substrate oxidation obtained with two or more antibodies	a) More than one P450 is involved in metabolism of the given substrate. Inclusion of both inhibitory antibodies should produce more extensive (nearly additive) inhibition.

6. References

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CYP2C8 and CYP2C9 mAb are covered under
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