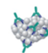
 

CYP IMMUNOINHIBIT KIT

PRODUCT# Hu-A011A, Hu-A011B

Please visit our website at: http://cyp450-gp.com to confirm that you have the most current version of this manual. Contact CYP450-GP sales (sales@cyp450-gp.com) regarding questions pertaining to this kit.

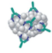
1. **Description**

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[1], 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 are included in the incubation mixture;
B) the drug-metabolizing reaction being tested for inhibition is catalyzed predominantly by the P450 targeted by the antibody. In 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.

[1] Additional analysis of P450 metabolic inhibition by polyclonal antibodies and mAb can be found at https://www.cyp450-gp.com/protocols.



2. **Components**

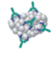
The polyclonal antibodies included in the *CYP ImmunoInhibit Kit* have been developed in rabbits using purified native or recombinant human CYP1A2, CYP2C19, CYP2D6 or CYP3A4 as immunogens. mAb-containing ascites fluid was produced in mice using hybridomas derived from animals immunized with recombinant human CYP2B6, CYP2C8 or 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 lyophylization from 100 mM potassium phosphate buffer (pH 7.4) containing 150 mM KCl.

3. **Storage and Reconstitution of Antibodies**

Store *CYP ImmunoInhibit Kit* containing the lyophylized IgGs at -20°C. Reconstitute by adding 100 µl (for 1 mg kit) or 200 µl (for 2 mg kit) of 100 mM potassium phosphate buffer, pH 7.4 (or other suitable buffer) to each vial of polyclonal IgG, and mix gently until powder dissolves, giving a final antibody concentration of 10 mg IgG/ml. Preimmune IgG should be reconstituted in 250-500 µl buffer to achieve the equivalent antibody concentration. CYP2B6, CYP2C8 and CYP2C9 mAb IgGs are reconstituted in the same manner but the final antibody concentration will be 1 mg IgG/ml. The antibody solutions should be stored at -20°C, avoiding 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 *O*-Deethylase	>80% at 3.0 mg IgG/mg HLMx
Anti-CYP2B6 (Hu-A012)	100-200 µg [White Cap]	Bupropion Hydroxylase	>85% at 0.075 mg IgG/mg HLMx
Anti-CYP2C8 (Hu-A004)	100-200 µg [Blue Cap]	Paclitaxel 6α-Hydroxylase	>85% at 0.04 mg IgG/mg HLMx
Anti-CYP2C9 (Hu-A003)	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]	*S*-mephenytoin 4'-Hydroxylase	>90% at 1.0 mg IgG/mg HLMx
Anti-CYP2D6 (Hu-A009)	1-2 mg [Green Cap]	Dextromethorphan *O*-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 [Clear 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

	ANTIBODY						
P450 ENZYME	**Anti-CYP1A2**	**Anti-CYP2B6**	**Anti-CYP2C8**	**Anti-CYP2C9**	**Anti-CYP2C19**	**Anti-CYP2D6**	**Anti-CYP3A4**
CYP1A2	+++	0	0	0	0	0	0
CYP2B6	0	+++	0	0	0	0	0
CYP2C8	0	0	+++	0	0	0	0
CYP2C9	0	0	0	+++	0	0	0
CYP2C19	0	0	0	0	+++	0	0
CYP2D6	0	0	0	0	0	+++	0
CYP3A4	0	0	0	0	0	0	+++
CYP3A5	0	0	0	0	0	0	++
CYP4A11	0	0	0	0	0	0	0

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

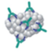
4. **Antibody Inhibition Assays**

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 subsequently be performed to reveal the overall extent of CYP2D6 involvement in this reaction. The IgG:microsomal protein ratio giving optimal inhibition of dextrorphan formation can then 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 H_2O
C) 10 mM NADPH - Prepare fresh by dissolving 5.6 mg in 0.6 ml Reagent A
D) Acetonitrile

II. Assay Format

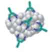
Tube #	HLMx	Antibody	NADPH	Other Rx Components
1	2.5 µl	10 µl **A**	--	+
2	2.5 µl	10 µl **A**	--	+
3	2.5 µl	10 µl **A**	--	+
4	2.5 µl	10 µl **A**	+	+
5	2.5 µl	10 µl **A**	+	+
6	2.5 µl	10 µl **A**	+	+
7	2.5 µl	10 µl **B**	+	+
8	2.5 µl	10 µl **B**	+	+
9	2.5 µl	10 µl **B**	+	+
10♦	2.5 µl	10 µl **A** + 2 µl **C**	+	+
11	2.5 µl	10 µl **A** + 2 µl **C**	+	+
12	2.5 µl	10 µl **A** + 2 µl **C**	+	+
13	2.5 µl	10 µl **A** + 2 µl **D**	+	+
14	2.5 µl	10 µl **A** + 2 µl **D**	+	+
15	2.5 µl	10 µl **A** + 2 µl **D**	+	+
16	2.5 µl	10 µl **A** + 4 µl **E**	+	+
17	2.5 µl	10 µl **A** + 4 µl **E**	+	+
18	2.5 µl	10 µl **A** + 4 µl **E**	+	+
19	2.5 µl	10 µl **F**	+	+
20	2.5 µl	10 µl **F**	+	+
21	2.5 µl	10 µl **F**	+	+
22	2.5 µl	10 µl **G**	+	+
23	2.5 µl	10 µl **G**	+	+
24	2.5 µl	10 µl **G**	+	+
25	2.5 µl	10 µl **H**	+	+
26	2.5 µl	10 µl **H**	+	+
27	2.5 µl	10 µl **H**	+	+

HLMx (pool of 50) = 20 mg protein/ml; 2.5 µl = 50 µg protein

A: Preimmune IgG, 10 mg IgG/ml; **B:** Anti-CYP1A2 IgG, 10 mg IgG/ml; **C:** Anti-CYP2B6 mAb IgG, 1 mg IgG/ml; **D:** Anti-CYP2C8 mAb IgG, 1 mg IgG/ml; **E:** Anti-CYP2C9 mAb IgG, 1 mg IgG/ml; **F:** Anti-CYP2C19 IgG, 10 mg IgG/ml; **G:** Anti-CYP2D6 IgG, 10 mg IgG/ml; H**:** 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 10-18 in order to keep the antibody concentration constant among assay tubes, thereby avoiding non-specific immunoglobulin effects.

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 27 tubes is needed to test each antibody in triplicate with the inclusion of 3 tubes without the cofactor NADPH.

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.

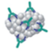
4. Add remaining components to the tubes, taking into account the prior addition of 50 µl assay buffer. These include the assay buffer (reagent A) and substrate (reagent B, 100 µM final concentration).

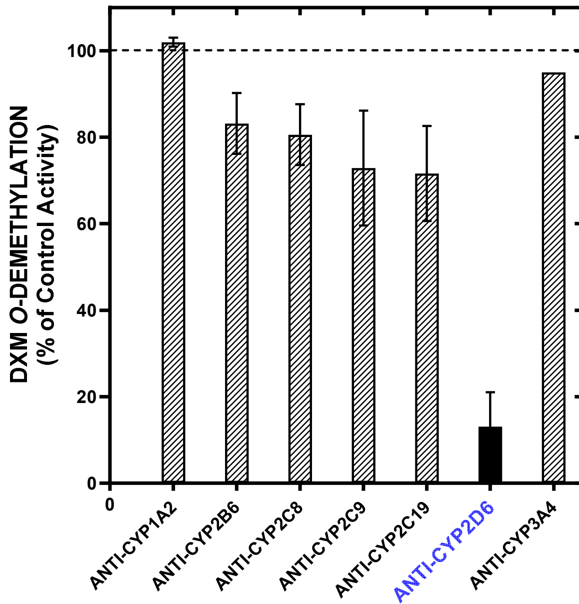
5. Start reactions with 25 µl of 10 mM NADPH (reagent C), and incubate for 15 min at 37°C. Omit NADPH from negative controls (tubes 1-3).

6. Terminate reactions with 100 µl of acetonitrile (reagent D), and analyze for the *O*-demethylated metabolite dextrorphan [5-6].

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 below, only CYP2D6 antibodies elicited marked (87%) inhibition of microsomal DXM *O*-demethylation while the other six *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.



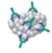


P450 METABOLISM IMMUNOTITRATION ASSAY

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

Tube	HLMx	Antibody	NADPH	Other Rx Components
1	2.5 µl	15 µl **A**	--	+
2	2.5 µl	15 µl **A**	--	+
3	2.5 µl	15 µl **A**	--	+
4	2.5 µl	15 µl **A**	+	+
5	2.5 µl	15 µl **A**	+	+
6	2.5 µl	15 µl **A**	+	+
7	2.5 µl	13 µl **A** + 2 µl **B**	+	+
8	2.5 µl	13 µl **A** + 2 µl **B**	+	+
9	2.5 µl	13 µl **A** + 2 µl **B**	+	+
10	2.5 µl	10 µl **A** + 5 µl **B**	+	+
11	2.5 µl	10 µl **A** + 5 µl **B**	+	+
12	2.5 µl	10 µl **A** + 5 µl **B**	+	+
13	2.5 µl	5 µl **A** + 10 µl **B**	+	+
14	2.5 µl	5 µl **A** + 10 µl **B**	+	+
15	2.5 µl	5 µl **A** + 10 µl **B**	+	+
16	2.5 µl	15 µl **B**	+	+
17	2.5 µl	15 µl **B**	+	+
18	2.5 µl	15 µl **B**	+	+

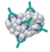
HLMx (pool of 50) = 20 mg protein/ml; 2.5 µl = 50 µg protein

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 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.**

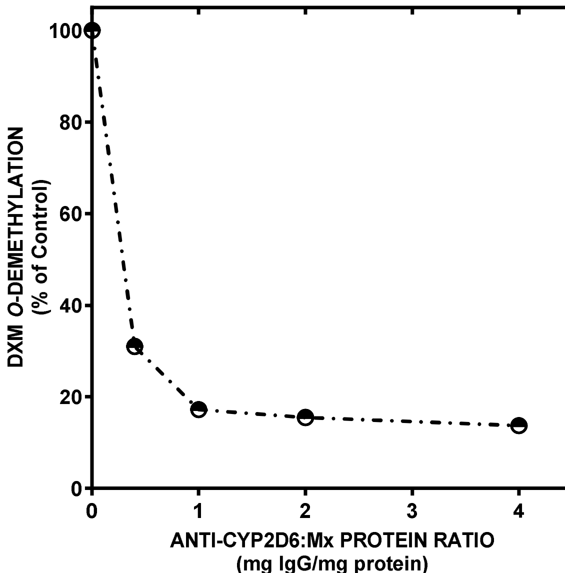
2. Add the above-described amounts of immune-specific and/or preimmune IgG to the tubes. Then, add liver microsomes (equivalent to 50 µg protein). A minimum of 18 tubes is needed to test each antibody: microsomal protein ratio.

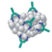
3. Incubate tubes **for 3 min @ 37°C** with shaking, then let stand for **10 min at room temp.** Finally, place tubes back onto ice.

4. Add the remaining components to the tubes, taking into account the prior addition of 50 µl assay buffer. These include the additional 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 µl of acetonitrile (reagent D), and analyze for the *O*-demethylated metabolite dextrorphan [5-6].





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 above indicate that CYP2D6 is the major, if not exclusive, catalyst of DXM *O*-demethylation in human liver.

5. **Troubleshooting**

SYMPTOM	CAUSE
CYP ImmunoInhibit Kit 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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 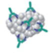

CYP2B6, CYP2C8 and CYP2C9 mAb are covered under
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