# Preparation of soluble PAP complexes

WOLF D. KUHLMANN

*Division of Radiooncology, Deutsches Krebsforschungszentrum, D-69120 Heidelberg*
*Laboratory Diagnostics & Cell Science, 56112 Lahnstein*

---

## Principle

In the original preparation procedure for PAP complexes, specific immune precipitates were first obtained by mixing anti-peroxidase with peroxidase (HRP). Then, precipitates were washed in saline, followed by resuspension in saline containing four times the amount of HRP used for precipitation and acidified to pH 2.3. After subsequent neutralisation and removal of undissolved complexes, soluble PAP complexes were finally obtained and purified from free HRP by ammonium sulfate precipitation.

## Protocol of PAP complex preparation

Highly purified peroxidase preparations from horseradish (HRP) with RZ 3 are employed throughout; Reinheitszahl RZ = $A_{403\ nm} : A_{275\ nm}$. These preparations are highly active, approx. 250 units/mg, measured with $H_2O_2$ and guaiacal. For experimental details in the preparation of hyperimmune sera see STERNBERGER LA (1979), STERNBERGER LA et al. (1970) and KUHLMANN W.D. (1984).*

## 1. Determination of the equivalence zone

The first step needed in the preparation of soluble PAP complexes is the determination of the equivalence zone (the *AgX* equivalence proportion of HRP is needed for subsequent calculation of HRP in the initial precipitation step of anti-HRP immune serum). Principles of immunological precipitation of antigens by antibodies have been described earlier (HEIDELBERGER M. and KENDALL F.E. 1935).

*Antigen solution*

Solutions of HRP from 0.05 mg HRP/mL saline to “x” mg HRP/mL (up to 3 mg/mL) are made up in increasing steps e.g. 0.05 mg/mL per tube.

*First test series*

0.2 mL of each diluted antigen step is added to numbered tubes; 0.2 mL of rabbit anti-HRP immune serum is then added to each tube and mixed. Tubes are left at 4ºC overnight and then centrifuged for 30 min at 5.000 rpm/min. Supernatants are collected invidually in tubes of a *Second test series* (numbers corresponding to numbers of the

---

* Chemicals for the preparation of PAP complexes and for peroxidase measurements can be toxic. They must be handled with care

first series) and 0.15 mL of each supernatant transferred into numbered tubes of *Third test series*.

*Second test series*

0.1 mL of anti-HRP is added to each tube. Precipitation will occur when the supernatants of the *First test series* still contain antigen. The presence of excess antigen in these supernatants is established by qualitative examination of formed precipitates after 1 h at room temperature.

*Third test series*

0.1 mL of 0.1 mg HRP/mL saline is added to each tube. Precipitation will occur when supernatants of the First test series still contain antibodies. The presence of excess antibody in these supernatants is established by qualitative examination of formed precipitates after 1 h at room temperature.

*Evaluation of tests*

Readings are performed with tubes containing:

(a) supernatants in the zone of excess antibody (observed in the *Third test series*);

(b) supernatants in the zone of excess antigen (observed in the *Second test series*);

(c) supernatants in the equivalance zone with no excess antibody or antigen (observed in *both test series*).

The concentration of HRP per mL Antigen solution (i.e. “Solutions of HRP from 0.05 mg/mL saline to “x” mg HRP/mL saline”) is determined in that tube whose equivalence zone is nearest to the antigen excess zone.

## 2. PAP preparation

PAP is preferably prepared by precipitation of anti-HRP immune serum with about 1.5 times of *AgX* equivalence proportion.

*Example of PAP preparation*

24 mg HRP may represent 1.5 times *AgX*, thus 16 mg is the *AgX* equivalence proportion.
When in the preliminary test the *AgX* is recorded in the tube in which 0.4 mg HRP per mL was added to the immune serum, then a total of 40 ml of anti-HRP is needed to precipitate 16 mg of HRP at the equivalence proportion or 24 mg at 1.5 times *AgX* equivalence proportion.

*PAP preparation*

6 mL HRP solution (= 24 mg HRP) is mixed with 40 mL anti-HRP immune serum. After 1 h at room temperature, precipitates are collected by centrifugation for 20 min at 4ºC and resuspended (forced through pipette) in small volume of cold 0.15 mol/L NaCl, then washed by addition of 100-200 mL 0.15 mol/L NaCl; three washing steps are performed each followed by centrifugation.

Precipitates are carefully resuspended at room temperature in 24 mL of 0.4% HRP in 0.15 mol/L NaCl. The mixture is acidified under continuous stirring to pH 2.3 by addition of HCl followed by neutralisation to pH 7.2.-7.4 with NaOH; add 2.4 mL acetate solution (0.08 mol/L sodium acetate and 0.15 mol/L ammonium acetate). This mixture is cooled to 0-4ºC and centrifuged in the cold for 10 min at 15.000-20.000 rpm; the supernatant is collected. All subsequent steps are carried out at 0-4ºC.

The supernatant is mixed with equal amounts of chilled saturated ammonium sulfate and stirred for 30 minutes. The product is centrifuged as in the above step and washed once with ammonium sulfate at half saturation. After centrifugation, the precipitate is dissolved in 24 mL A. dest. and dialysed extensively against large volumes of sodium ammonium acetate saline. Remaining precipitates are centrifuged as above and the supernatant is collected. PAP complexes are in the supernatant.

HRP and anti-HRP contents in soluble PAP complexes can be determined by absorbance measurements in the spectrophotometer at $A_{400\ nm}$ and $A_{280\ nm}$, respectively. The average molar ratio of HRP to anti-HRP will usually be 3:2.

*© Prof. Dr. Wolf D. Kuhlmann* *10.09.2006*