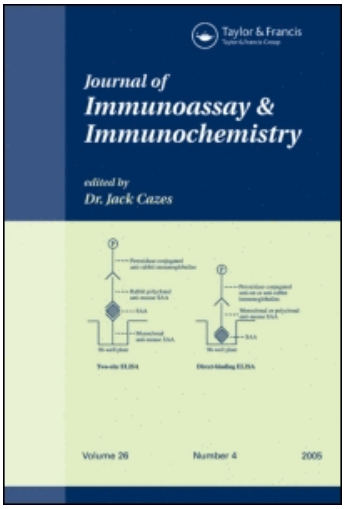


## Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

# Generation of Polyclonal Antibodies Against Recombinant Human Activin a

James A. Lofgren[a]; Ralph Schwall[b]; Charles Schmelzer[c]; Wai Lee T. Wong[a]
[a] Departments of Immunology Research & Assay Technology, South San Francisco, California [b] Developmental Biology, South San Francisco, California [c] Process Recovery, Genentech, Inc., South San Francisco, California

**To cite this Article** Lofgren, James A. , Schwall, Ralph , Schmelzer, Charles and Wong, Wai Lee T.(1991) 'Generation of Polyclonal Antibodies Against Recombinant Human Activin a', Journal of Immunoassay and Immunochemistry, 12: 4, 565 — 578
**To link to this Article: DOI:** 10.1080/01971529108053280
**URL:** http://dx.doi.org/10.1080/01971529108053280

JOURNAL OF IMMUNOASSAY, 12(4), 565-578 (1991)

# GENERATION OF POLYCLONAL ANTIBODIES AGAINST RECOMBINANT HUMAN ACTIVIN A

James A. Lofgren,* Ralph Schwall,‡ Charles Schmelzer§ and Wai Lee T. Wong*

Departments of *Immunology Research & Assay Technology,
‡Developmental Biology, and §Process Recovery,
Genentech, Inc., 460 Point San Bruno Boulevard,
South San Francisco, California 94080



## ABSTRACT

A goat antiserum to purified recombinant human activin A (rhAct-A), a dimer formed by two $\beta_A$-subunits of inhibin, has been produced. The immunoreactivity of the antiserum has been evaluated in an antigen coated enzyme-linked immunosorbent assay, in a radioimmunoassay using iodinated rhAct-A, and by Western blot analysis. The antiserum demonstrated some cross reactivity to inhibin A, a structurally related heterodimer which contains an identical $\beta_A$-subunit coupled to a distinct, though similar, $\alpha$ subunit. A simple radioimmunoassay for rhAct-A in tissue culture supernatant has been developed with rhAct-A affinity column purified polyclonal antiserum. The assay is precise and sensitive with a range of 0.31-40 ng/ml. The cross reactivity of inhibin A in the RIA is about 4.3%. Despite its cross-reactivity this antiserum will facilitate studies of the physiology of activin A and inhibin A which includes a Western blot analysis where a molecular size distinction is accomplished.

(KEY WORDS: activin A antiserum; iodination of activin A; activin A radioimmunoassay)

---

Address requests for reprints to: Dr. W. L. Wong, Department of Immunology Research and Assay Technology, Genentech Inc., 460 Pt. San Bruno Boulevard, South San Francisco, California 94080.

## INTRODUCTION

Activin is a polypeptide hormone that stimulates the secretion of follicle stimulating hormone (FSH) (1) and enhances erythroid differentiation (2, 3). It is a 24 kD protein composed of two inhibin β-subunits that are linked by disulfide bonds (4-6). Ovarian follicular fluid contains two distinct forms of activin; activin AB is a heterodimer of $\beta_A$ and $\beta_B$-subunits (4, 5), activin A is a homodimer of $\beta_A$-subunits (6). In addition, Mason *et al.* have produced activin B ($\beta_B$-$\beta_B$) by recombinant expression (7). All three forms have similar activities and potencies (4-7).

Our understanding of the physiology of activin has been hampered by the lack of reagents to study its secretion. This is due partly to the limited amounts of material available from native sources. To circumvent this problem, several laboratories have produced antisera to small synthetic peptides that correspond to portions of the deduced amino acid sequences (8). While such antisera are useful, the vast majority of anti-peptide antisera cross-react only weakly with the protein from which the sequence was derived.

It has recently become possible to produce activin A by recombinant DNA technologies (9), which have allowed for the production of quantities sufficient for immunizations. Initial attempts at producing antisera in rabbits and mice were fruitless, probably owing to the extremely high degree of sequence conservation across species and the possible immunosuppressant effects of activin A (10-13). We report the first successful generation of an antiserum to recombinant human activin A (rhAct-A) and describe the production, characterization and application of this antiserum as an analytical reagent.

## MATERIALS AND METHODS

### Recombinant Human Activin A and Standards

rhAct-A was obtained by its expression in human kidney 293 cells at Genentech, Inc. The protein was purified by sequential gel filtration, cation-exchange chromatography and C18 reversed-phase HPLC (9). SDS-PAGE analysis of the purified rhAct-A revealed a single silver stained band at $M_r$ 26,000. Amino terminal sequence analysis of the purified rhAct-A was consistent with the expected amino terminus of the β-subunit of inhibin A deduced from DNA sequence. Protein content was determined by quantitative amino acid analysis. The rhAct-A utilized for standard was stored at -20°C. All standards were prepared fresh daily by diluting into isotonic phosphate-buffered saline (PBS; pH 7.2) containing per liter 5 g bovine serum albumin (BSA), 1 g of sodium dodecyl sulfate (SDS), 0.5 ml of polysorbate (Tween-20) and 0.1 g thimerosal (assay buffer).



### Miscellaneous Reagents

Recombinant human growth hormone (rhGH), recombinant human inhibin A (rhInh-A), recombinant human activin B (rhAct-B), and recombinant human transforming growth factor-β (rhTGF-β) were obtained and cloned at Genentech, Inc. Human follicle-stimulating hormone (FSH), human luteinizing hormone (LH), and human thyroid hormone (TSH) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Porcine follicular fluid was obtained from J.R. Scientific (Woodland, CA.).

### Production of Antiserum

A pair of six-month-old Nubian goats, one male and one female, and a pair of female New Zealand White rabbits, were each immunized with 100 μg of rhAct-A

in complete Freund's adjuvant equally divided between intradermal and intramuscular injections. After a three week rest period, the goats were boosted with 50 μg of immunogen in Freund's incomplete adjuvant equally divided between subcutaneous and intramuscular immunizations. The animals were bled the following week and tested for antibody titers by rhAct-A coated ELISA. A weekly schedule of alternating boosts and bleeds was continued for two months until an adequate antibody titer developed. Thereafter, the animals were boosted every two weeks and eventually switched to monthly injections when the desired titers were reached.



Antiserum Screen ELISA

Sera obtained from the immunized goats were screened by ELISA for antibody to rhAct-A on 96-well polystyrene assay plates (Maxisorb, Nunc, Kamstrup, Denmark) coated for 20 h at 4°C with 100 μl per well of 1 μg /ml purified rhAct-A in 50 mmol/liter carbonate buffer, pH 9.6 (coat buffer). Nonspecific binding sites on the coated plates were blocked with a PBS solution containing 5 g/liter of BSA (blocking buffer) for 1 h at room temperature. Crude bleeds were first diluted 1:100 in PBS containing per liter 5 g BSA, 0.5 ml Tween-20 and 0.1 g Thimerosal (dilution buffer). After washing the plates with wash buffer (0.5 ml of Tween-20 and 0.1 g Thimerosal per liter of PBS), 100 μl of each serially diluted bleed was added to each well and incubated for 2 h at ambient temperature. After the plates were washed, bound antibodies were detected with donkey anti-goat IgG conjugated to horseradish peroxidase (Immunosearch, Emeryville, CA) incubated for 1 hr at ambient temperature. The plates were washed again, and 100 μl of freshly prepared substrate solution (0.4 g of *o*-phenylene-diamine dihydrochloride in one liter of PBS plus 0.4 ml of 30% hydrogen peroxide) was added to each well and the plates were incubated in the

dark for 30 min at room temperature. The reaction was stopped with 2.25 mol/liter sulfuric acid, 100 µl per well, and the absorbance of each well at 490 nm ($A_{490}$nm) was determined spectrophotometrically with a Vmax plate reader (Molecular Devices, Palo Alto, CA). Resulting absorbance was plotted against the log dilutions and the titer of a bleed is expressed as the dilution giving 50% of its maximum absorbance. Bleeds that have a titer greater than 10,000 were selected and tested in a radioimmunoassay.

Iodination of rhAct-A

A modified sequential chloramine T iodination was performed. To 5 µg of rhAct-A in PBS, containing 1 mCi of $NaI^{125}$, 20 µl of freshly prepared chloramine T at 0.1 g/liter was added three times in succession, with gentle mixing, and incubated for 2.0, 1.5, and 1.0 min. The reaction was quenched by adding 20 µl of 50 mmole/liter n-acetyl tyrosine and 20 µl of 1M potassium iodide. The volume was brought to 200 µl with PBS and the iodinated protein was loaded on a PD-10 column (Pharmacia, Uppsala, Sweden), prewashed with 6 volumes of PBS containing per liter 5 g BSA, and eluted with the wash buffer. The specific activity was determined to be between 50 to 60 µCi/µg by using trichloroacetic acid precipitation and assuming that 100% of the rhAct-A was iodinated. The specific activity was determined to be 57 µCi/µg by trichloroacetic acid precipitation.

Antiserum Evaluation by Radioimmunoassay

Sera obtained was serially diluted 1:3 from a initial dilution of 1:100 in assay buffer. To 100 µl of diluted serum in 12 x 75 mm polystyrene test tubes, 100 µl of 20 ml per liter normal goat serum (Pel Freez, Rogers, AR) in assay buffer was added and the tubes were vortexed. Radioiodinated rhAct-A (20,000 cpm per 100 µl)was added, and the vortexed tubes were incubated overnight (16 hr) at

ambient temperature. The next day, 40 µl of donkey anti-goat IgG (Pel Freez, Rogers, AR) was added, the tubes were mixed by vortexing and incubated for 1 hour at ambient temperature. After incubation, 1 ml of cold PBS was added, the tubes were vortexed and centrifuged for 20 min at 4°C in a Beckman J6 at 2000 x g. The centrifuged samples were then decanted, blotted on absorbant paper and counted in a LKB series 1277 gamma counter for 1 minute. Results were plotted as percent of tracer bound against the dilution of antiserum.

Antibody Purification

Bleeds from goat 290B with similar percent bound tracer were pooled and partially purified by delipidization, defibrinization, and dialysis against PBS (pH 7.4). The antibody was then precipitated with an equal volume of saturated ammonium sulfate, and dialyzed extensively against PBS. Next it was loaded onto a rhAct-A coupled affigel-10 affinity column, washed with 0.5 M NaCl in PBS, and eluted with 0.2 M glycine-HCL (pH 2.4). The specific IgG eluate was neutralized immediately with 1 M Tris-HCl (pH 10), dialyzed against PBS and then stored aliquoted at -20°C.

Radioimmunoassay

A sequential tracer addition radioimmunoassay was employed using polystyrene 12 x 75 mm conical tubes (Sarstedt, W. Germany). Standards and samples, diluted if necessary, in assay buffer were added to each tube (100 µl per tube) followed by 100 µl/ tube of antiserum diluted 1:2000. The samples and antiserum were vortexed and incubated overnight at 4°C (>18 hr). rhAct-A tracer was added at 20,000 cpm per tube and the incubation was continued at room temperature for 4 hr. Next 25 µl of second antibody, donkey anti-goat IgG (Pel Freez, AR) was added, the tubes were vortexed and incubated for an additional

30 min. After the addition of 1 ml of cold PBS, the tubes were centrifuged at 2000 x g for 20 min, decanted and counted for 1 min in a gamma counter. The standard curve was generated by a semi-logarithmic plot of percent tracer bound against the concentration of unlabeled rhAct-A.

Western Blot

Samples were prepared as follows: purified rhAct-A and rhAct-B were produced as previously described (7,9), and conditioned media containing both rhInh-A and rhAct-A were obtained from a cell line transfected with a mixture of $\alpha$ and $\beta_A$ cDNAs (14). One volume of each sample was mixed with 2 volumes of 3X SDS-PAGE sample buffer and heated at 100°C for 5 min. Porcine inhibin was partially purified from follicular fluid by ammonium sulfate precipitation followed by hydrophobic interaction chromatography on phenyl-Sepharose (15). The inhibin concentration in this preparation was approximately Four micrograms per milliliter, as determined by bioassay (assuming an ED50 of ~0.5 ng/ml for purified inhibin, ref. 16). One milliliter of this preparation was diluted with 1 ml of 40% acetonitrile in 0.2% TFA and then loaded onto a Sep-Pak C18 column. The column was washed with 10 ml 20% acetonitrile, 0.1% TFA and eluted with 1 ml 50% acetonitrile, 0.1% TFA. The eluate was dried in a Speed-Vac (Savant) and dissolved in 100 µl sample buffer and then heated at 100°C for 5 min.

Samples were loaded onto a 12.5% polyacrylamide gel (0.75 mm thick) and electrophoresed at 200 V for 40 min, using a BioRad Mini-Protein II apparatus. The gel was equilibrated in transfer buffer (25 mmole/liter Tris, 192 mmole/liter glycine, 200 ml/liter methanol), and then loaded into a BioRad Mini-Transblot Transfer Cell. Transfer was performed at 100 V for 1 hr, with cooling. The nitrocellulose blots were removed from the transfer unit and immediately blocked in PBS containing 0.5 ml/liter of Tween-20 and 30 g/liter of BSA for 1 hr. The

blocking and all subsequent steps were carried out at room temperature on an orbital shaker. The blocked strips were incubated overnight in anti-activin A antiserum or normal goat serum, both diluted 1:2000 in PBS with 0.5 ml/liter Tween-20 and 1 g/liter BSA. The blots were then washed 3 times (5 min each) in PBS, plus 0.5 ml/liter Tween-20. Detection of bound antibody was achieved by incubating for 2 hr in alkaline phosphatase-anti-goat IgG (Sigma, 1:1000 in PBS 10 ml/liter normal rabbit serum), After washing with PBS, 0.5ml/liter NP 40 for 10 min and PBS, 0.5 ml/liter Tween-20, the blot was rinsed once in PBS, once in 100 mmole/liter CHES buffer (2-[N-cyclohexylamine] ethane-sulfonic acid) pH 9.6, and then developed in a substrate solution consisting of 40 ml 100 mmol CHES buffer (pH 9.6), 300 µl 100 g/liter magnesium chloride, 20 µl 75 g/liter nitroblue tetrazolium, and 300 µl 25 g/liter 5-bromo-4-chloro-3-indolyl phosphate.



## RESULTS AND DISCUSSION

Two rabbits and two goats were immunized with rhAct-A. Both of the rabbits and one of the goats responded poorly to the immunization and developed ELISA titers of only 2,500 $\pm$ 500 (Fig. 1). However, one of the goats (#290B) showed an ELISA titer of 300,000. In the radioimmunoassay, 290B bound greater than 80% of the tracer, while the other antisera bound, at best, 25% (data not shown). Subsequently, only the antiserum from 290B was purified and used as the immunoreagent for RIA development and gel analysis work.

A radioimmunoassay was developed using affinity purified goat anti-rhAct-A (290B) and radioiodinated rhAct-A. The RIA had a sensitivity of 0.2 ng/ml, corresponding to 2 SD lower than the mean percent bound in 20 zero standard samples. As shown in Fig. 2, the working range of the assay was 0.31 to 20 ng/ml. The precision of the assay was tested at three different rhAct-A

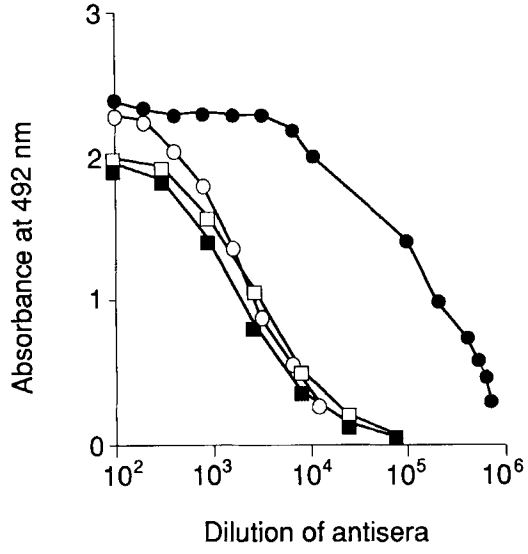


FIGURE 1. Determination of antiserum titers by ELISA. Serial dilution of sera from goat 290A (○—○), goat 290B (●—●), rabbit 903 (▲—▲) and rabbit 904 (△—△) were assayed in rhAct-A coated polystyrene microtiter plates using peroxidase-conjugated secondary antibody. Results were expressed as the absorbance at 492 nm.

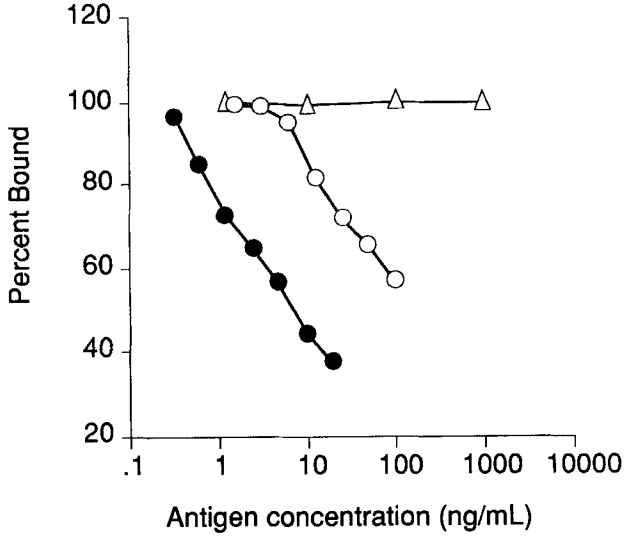


FIGURE 2. RIA standard curve for rhAct-A (●—●), for rhInh-A (○—○) and rhAct-B (△—△).

TABLE 1
PRECISION of RIA

| A. Intra-Assay Precision | Samples 1 | 2 | 3 |
|---|---|---|---|
| Replicates | 20 | 20 | 20 |
| Mean (ng/ml) | 0.78 | 8.40 | 31.82 |
| S.D. (ng/ml) | 0.05 | 0.87 | 3.11 |
| C.V. (%) | 6.41 | 10.36 | 9.77 |
| B. Inter-Assay Precision | | | |
| | 1 | 2 | 3 |
| Replicates | 10 | 10 | 10 |
| Mean (ng/ml) | 0.75 | 9.97 | 36.40 |
| S.D. (ng/ml) | 0.08 | 1.15 | 5.12 |
| C.V. (%) | 10.70 | 11.53 | 14.07 |



TABLE 2
SPECIFICITY of ANTISERUM by RIA

| Cross-reactant | Concentration Tested (μg/ml) | Assayed (ng/ml) | Cross-reactivity (%) |
|---|---|---|---|
| FSH | 1.0 | 0.98 | 0.098 |
| Prolactin | 1.0 | 1.27 | 0.127 |
| LH | 1.0 | 0.39 | 0.039 |
| Insulin | 1.0 | 0.42 | 0.042 |
| rhTGF $\beta_1$ | 10.0 | 0.21 | 0.002 |
| rhGH | 1.0 | 0.14 | 0.014 |
| rhAct B | 100.0 | 0.30 | 0.001 |
| rhInh-A | 0.1 | 4.28 | 4.300 |

concentrations, and in all instances the coefficients of variation were less than 15.7% (Table 1). The assay exhibited 4.3% cross-reactivity with recombinant rhInh-A (Fig. 2 ) but less than 0.2% cross-reactivity with rh Act-B (Fig. 2) and a variety of other protein hormones (Table 2). This cross-reactivity observed with rhInh-A may be enhanced by the presence of 1 g/liter SDS in the RIA assay buffer.

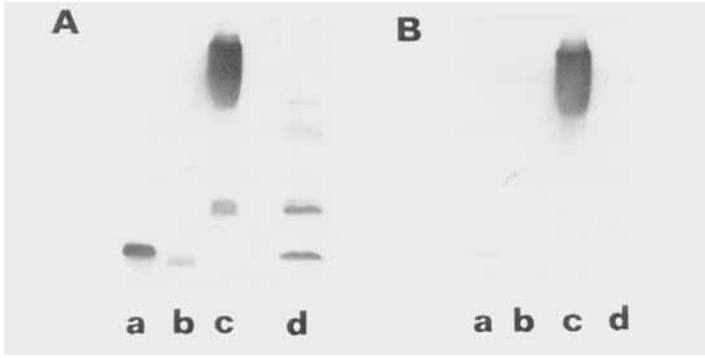


FIGURE 3. Western blot analysis of rhAct-A and rhInh-A by antiserum 290B. Samples were separated by electrophoresis in a 12.5% polyacrylamide gel and transferred to nitrocellulose. Duplicate blots were incubated in antiserum 290B (left panel, A) or normal goat serum (right panel, B) followed by alkaline phosphatase conjugated anti-goat IgG. Lane a, 50 ng rhAct-A; lane b, 50 ng rhAct-B; lane c, partially purified porcine follicular fluid; lane d, conditioned medium from cell line expressing a mixture of rhAct-A and rhInh-A.



However, the inclusion of SDS at 1 g/liter is essential to expose the epitopes on rhAct-A in order that 290B can bind, and make the RIA possible.

The specificity of the antiserum was further tested by Western blot analysis (Fig. 3). Intact rhAct-A was readily detected in such blots (lane a). An equivalent mass of rhAct-B was detected to a lesser extent (lane b). By comparing intensities at a variety of protein loads, it is estimated that the cross-reactivity with rhAct-B under these conditions is ~5% (not shown). Two major bands of ~24 kD and ~32 kD were observed in conditioned media from a cell line expressing a mixture of rhAct-A and rhInh-A (lane d). The fact that these bands were of equal intensity in the blots and also in silver-stained gels suggests that the antibody reacts with both molecules equally. This at first may appear to contrast with the 4.3% inhibin A and 0.001% rhAct-B cross-reactivity observed in the RIA, but this cross-reactivity was measured under conditions of limiting antibody concentration, whereas blotting is conducted in antibody excess.

In a crude preparation of porcine follicular fluid (pFF), a doublet was detected (lane c). The lower band of the doublet comigrated with rhInh-A, and the upper band may represent a glycosylation variant of rhInh-A. A large smear was observed near the top of the gel in the lane loaded with pFF, which represents nonspecific binding of the second antibody since it occurred when normal goat serum was substituted for 290B (Fig. 3B) or when the primary antibody was omitted altogether. As shown in Fig. 3B, none of the bands detected in lanes a, b, or d were apparent when blots were incubated in normal goat serum (Fig. 3B). However, there was an extremely faint band in lane a due to minor non-specific binding between goat serum and rhAct-A.



By RIA activin/inhibin (980 ng/ml) was detected in pFF, and from the Western blot shown in Fig. 3A it can be seen that this activity is primarily due to inhibin A. Since pFF is a major source for inhibin and there is a greater than 80% homology between porcine and human inhibin (1), it is not unexpected that the RIA can quantitate the concentration of inhibin/activin . Activin A may also be present, but was beyond the detection limit of the Western blot .

These results show that antisera can be successfully developed to activin, a molecule that exhibits extreme sequence conservation among species. The antiserum can be used in radioimmunoassays, but caution must be exercised because of a low but significant cross-reactivity with inhibin A . However, the relative amounts of inhibin and activin in a sample can be determined by Western blotting, in which inhibin and activin can be distinguished by size. This antiserum should prove to be extremely useful in delineating the regulation of activin and inhibin secretion.

## ACKNOWLEDGEMENTS

We thank the Cell Culture R & D Group at Genentech Inc. for the production of rhAct-A, and Tony Mason at Genentech for rhInh-A and rhAct-B. The authors also gratefully acknowledge the excellent laboratory assistance of Edith Matsuyama at Genentech.

## REFERENCES

1. De Jong, F.H. Inhibin. Physiological Reviews 1988; 68: 555-607.

2. Eto, Y., Tsuji, T., Takezawa, M., Takano S., Yokogawa, Y., Shiba, H. Purification and characterization of erythroid differentiation factor (EDF) isolated from human leukemia cell line THP-1. Biochem. Biophys. Res. Commun. 1987 142:1095-1103.

3. Yu, J., Shao, L., Lemas, V. et al. Importance of FSH-releasing protein and inhibin in erythrodifferentiation. *Nature* 1987; 330: 765-767.

4. Vale, W., Rivier, J., Vaughan, J. et al. Purification and characterization of an FSH releasing protein from porcine follicular fluid. Nature 1986; 321: 776-779.

5. Ling, N., Ying, S. Y., Ueno, N. et al. Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. Nature 1986, 321: 779-782.

6. Ling, N., Ying, S.-Y., Ueno, N. et al. A homodimer of the β-subunits of inhibin A stimulates the secretion of pituitary follicle stimulating hormone. Biochem. Biophys. Res. Commun. 1986; 138: 1129-1137.

7. Mason, A. J., Berkemeier, L. M., Schmelzer, C., Schwall, R. H. Activin B: precursor sequences, genomic structure and *in vitro* activities. Mol. Endocrinol. 1989; 3: 1352-1358.

8. Vaughan, J. M., Rivier, J., Corrigan, A. Z. et al. Detection and purification of inhibin using antisera generated against synthetic peptide fragments. Meth. Enzymol. 1989; 168: 588-617.

9. Schwall, R. H., Nikolics, K., Szonyi, E., Gorman, C., Mason, A. J. Recombinant expression and characterization of Human Activin A. Mol. Endocrinol. 1988; 2: 1237-1242.

10. Mason, A. J., Niall, H. D., Seeburg, P. H. Structure of two human ovarian inhibins. Biochem. Biophys. Res. Commun. 1986; 135: 957-964.

11. Mason, A. J., Hayflick, J. S., Ling, N. et al. Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-β. Nature 1985; 318: 659-663.

12. Forage, R. G., Ring, J. M., Brown, R. W. et al. Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. Proc. Natl. Sci. USA 1986; 83:3091-3095.

13. Esch, F., Shimasaki, S., Cooksey, K. et al. Complementary deoxyribonucleic acid (cDNA) cloning and DNA sequence analysis of rat ovarian inhibins. Mol. Endocrinol. 1987; 1:388-396.

14. Mason, A. J. Structure and recombinant expression of human inhibin and activin. In: Hodgen, G. D. et al, eds. Nonsteroidal Gondal Factors: Physiological Roles and Possibilities in Contraceptive Development. Norfolk: The Jones Institute Press, 1988: 19-29.

15. Jansen, E. H. J. M., Steenbergen, J., de Jong, F. H., van der Molen, H. J. The use of affinity matrices in the purification of inhibin from bovine follicular fluid. Mol. Cell. Endocrinol. 1981; 21: 109-117.

16. Rivier, J., Spiess, J., McClintock, R., Vaughan, J., Vale, W. Purification and partial characterization of inhibin from porcine follicular fluid. Biochem. Biophys. Res. Commun. 1985; 133: 120-127.