INTERNATIONAL JOURNAL FOR INNOVATIVE RESEARCH IN MULTIDISCIPLINARY FIELD ISSN: 2455-0620 Volume - 3, Issue - 11, Nov – 2017
Monthly, Peer-Reviewed, Refereed, Indexed Journal with IC Value: 86.87 Impact Factor: 5.60 Publication Date: 30/11/2017

Golden Dot, A Simple Approach for Antibody-Antigen Interaction Detection on Nitrocellulose Membrane, by Using Bt *Cry1Ac* , Polyclonal, Monoclonal Antibodies and Nano-Colloidal Gold

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Abstract: *A novel technique was developed for high-throughput screening of antigens, based upon the nano colloidal gold conjugated antibody and antigen interaction . A procedure for the assay is described as polyclonal and monoclonal antibodies were immoblized on nitrocellulose membrane and antigen mixed with nano colloidal gold conjugated specific antibodies were applied on that spots to determine interaction. In this study Cry1Ac protein was used as antigen. For its interaction Anti Bt Cry1Ac antibody was used as monoclonal antibody. Anti rabbit goat antibody (IgG) was used as polyclonal antibody as control. The method has the merit of being simpler in operation and more sensitive than comparable existing procedures. Its applications can be broad in primary screening of recombinant plants. With minor modifications it can be used in serum pathogen detection.*

Key Words: Antigens, Nano colloidal gold, Bt Cry1Ac, Polyclonal antibody, Anti rabbit goat antibody

1. INTRODUCTION:

From the Kiihler and Milstein's monoclonal antibody techniques[1] , these unique immunochemical agents have used in a broad variety of research fields. Furthermore, the use of labbelled antibodies gave a rapidly expanding sky for useful diagnostic procedures[2]. The need is to find out the specificity of an antibody against a complex mixture of protein antigens, this purpose leads to a two-dimensional solid-phase immunoassay system for proteins which are separated by electrophoreses on polyacrylamide gel and transffered on to nitrocellulose membrane[3] .An antigen-coated nitrocellulose has been also used to find and fix antibody-secreting cells growing in agar [4].

Solid-phase techniques based upon nucleic acid hybridization to nitrocellulose membrane - binded DNA also been widely used[5] . A current modification of the Southern blot system is the “dot blot-hybridization” method in which, instead of being blotted from a gel, the probe is applied directly to the nitrocellulose as a spot[4]. This permits a significant simplification of the procedure and also the instantaneous screening of a number of samples[6]. We describe here an comparable protocol for antigen-antibody interaction which we, by analogy, call “dot immunobinding or Golden immuno-dot” We show the application of the protocol to the screening of Cry1Ac of samples must be screened for a specific antibody, and as a detection procedure, by which large numbers of antibodies may be assayed simultaneously

2. MATERIAL AND METHODS:

For nanocolloidal gold “Gold-in–a-Box™ kit (Cat. No. NGIB01-B044) was procured from BioAssay Works, LLc., USA It contained Naked Gold®-(44ml)-20nm-15 OD particles, buffer solution A, buffer solution B, buffer solution C, buffer solution D, BSA blocking solution and gold drying buffer.

Highly purified polyclonal antibodies, which were Rabbit IgG (Cat. No. 41-GR30) and Bt Cry1Ac antibody (Cat. No. 70r-BR005) were purchased from Fitzgerald International, country. Bt Cry1Ac was in lyophilized form, while Rabbit IgG was supplied in phosphate buffered saline (PBS), pH 7.4 with 0.02% Na Azide. 1X PBS (10 mM), 5% sucrose solution, BSA blocking buffer (3% BSA in 10 mM PBS) and PBS-Tween 20 (3%) with 5% BSA and 2 ml polyvinyle alcohol (blocking buffer) were prepared separately.

2.1 IMMUNO-BLOT FOR THE DETERMINATION OF ANTIBODY AND ANTIGEN INTERACTION

An immuno-blot test was scheduled for the confirmation of antibody and antigen interaction. Following four different experiments were performed using Fermentas nitrocellulose membrane as reaction area.

- Cry1Ac antibody conjugated with nanocolloidal gold was dropped (3µl) on nitrocellulose membrane and nominated this as spot number 1.
- Bt cotton seeds were trampled in 1X SEB4 buffer (pH 10.5) , used its 2 µl supernatant as antigen (Cry1Ac protein) Source and mixed it with 3 µl Cry1Ac antibody conjugated with nanocolloidal gold. Total volume of 5 µl was dropped on nitrocellulose membrane and labeled it as spot number 2.
- Antibody IgG (goat anti rabbit antibody) a secondary antibody was dropped 1 µl on nitrocellulose membrane and dried at 37ºC for 30 minutes. After 30 minutes Bt cotton seeds were trampled in 1X SEB4 buffer (pH 10.5) , used its 2 µl supernatant as antigen (Cry1Ac protein) Source and mixed it with 3 µl Cry1Ac antibody conjugated with nanocolloidal gold. Total volume of 5 µl was dropped on nitrocellulose membrane at same place where IgG was dropped and labeled it as spot number 3.
- Anti-Cry1Ac Antibody (1 µl) was dropped nitrocellulose membrane and dried it at 37ºC for 30 minutes. After 30 minutes Bt cotton seeds were trampled in 1X SEB4 buffer (pH 10.5) , used its 2 µl supernatant as antigen (Cry1Ac protein) Source and mixed it with 3 µl Cry1Ac antibody conjugated with nanocolloidal gold. Total volume of 5 µl was dropped on nitrocellulose membrane at same place where Anti-Cry1Ac Antibody was dropped and labeled it as spot number 4.

2.2 IMMUNO- BLOT FOR THE DETECTION OF ANTIBODY AND ANTIGEN INTERACTION BY USING 1X PBS AS PROTEIN ANTIGEN EXTRACTION BUFFER

As protein axtraction buffer has a great importance during antige-Antibody interaction, Again an immuno-blot test was designed for the detection of antibody-antigen interaction. For this following four different experiments were performed by using Fermentas Nitro Cellulose Membrane as reaction area. Secondly, simple 1X PBS (10mM) buffer was used as protein extraction buffer.

- Non-Bt cotton seeds were trampled in 1X PBS buffer (pH 7.4), 2 µl of its supernatant was used and mixed it with 3 µl of nanocolloidal gold conjugated Cry1Ac antibody. Total volume of 5 µl was dropped on nitrocellulose membrane and labeled it as spot No. 1.
- Bt cotton seeds were trampled in 1X PBS buffer (pH 7.4) , 2 µl of its supernatant was used as source of antigen (Cry1Ac protein) and mixed with 3 µl of nanocolloidal gold conjugated Cry1Ac antibody. Total volume of 5 µl was dropped on nitrocellulose membrane and labeled it as spot No. 2.
- Antibody IgG (goat anti rabbit antibody) a secondary antibody was dropped 1 µl on nitrocellulose membrane and dried at 37ºC for 30 minutes. After 30 minutes Bt cotton seeds were trampled in 1X PBS buffer (pH 7.4), used its 2 µl supernatant as antigen (Cry1Ac protein) Source and mixed it with 3 µl Cry1Ac antibody conjugated with nanocolloidal gold. Total volume of 5 µl was dropped on nitrocellulose membrane at same place where IgG was dropped and labeled it as spot number 3.
- Anti-Cry1Ac Antibody (1 µl) was dropped nitrocellulose membrane and dried it at 37ºC for 30 minutes. After 30 minutes Bt cotton seeds were trampled in 1X PBS buffer (pH 7.4), used its 2 µl supernatant as antigen (Cry1Ac protein) Source and mixed it with 3 µl Cry1Ac antibody conjugated with nanocolloidal gold. Total volume of 5 µl was dropped on nitrocellulose membrane at same place where Anti-Cry1Ac Antibody was dropped and labeled it as spot number 4.

The nanocolloidal gold used to conjugate antibodies in these experiments was of pH 8.8.

2.3 VERIFICATION OF ANTIBODY ANTIGEN INTERACTION

For further verification that antigen (Cry1Ac protein) was binding specifically with primary Antibody (Anti-Cry1Ac antibody) another experiment was designed and performed. Two types of cotton seed samples were taken; one was Bt (Cry1Ac) positive and other was Bt (Cry1Ac) negative, ground them separately in 1X PBS and took their supernatants (extracted proteins). Took a piece of Fermentas NC membrane, marked four parts with lead pencil and proceeded as under.

- The secondary antibody, IgG (goat-anti-rabbit antibody) was dropped (1µl of 1 mg/ml) on NC membrane and dried at 37ºC for 30 minutes. Then 2 µl of protein extract from Bt cotton seeds was mixed with 3 µl of gold antibody conjugate (pH 8.8) and total 5 µl mix was put exactly on the spot of IgG.
- Secondary antibody, IgG (1 µl of 1 mg/ml) was spotted on NC membrane and dried at 37ºC for 30 minutes. Then 2 µl of protein extract from non-Bt cotton seeds was mixed with 3 µl of gold antibody conjugate (pH 8.8) and total 5 µl mix was put exactly on the spot of IgG.
- Anti-Cry1Ac antibody (1µl of 1 mg/ml) was spotted on NC membrane and dried at 37ºC for 30 minutes. Then 2 µl of protein extract from Bt cotton seeds was mixed with 3 µl of gold antibody conjugate (pH 8.8) and total 5 µl mix was put exactly on the spot of anti-Cry1Ac antibody.
- Anti-Cry1Ac antibody (1µl of 1 mg/ml) was spotted on NC membrane and dried at 37ºC for 30 minutes. Then 2 µl of protein extract from non-Bt cotton seeds was mixed with 3 µl of gold antibody conjugate (pH 8.8) and total 5µl mix was put exactly on the spot of anti-Cry1Ac antibody.

In all these experiments antibodies of both types were spotted first on membranes at the same times and then membranes were placed at 37ºC for drying and attachment of antibodies on NC membranes. Then other applications and treatments were made.

RESULTS:

3.1 IMMUNO-BLOT FOR THE DETERMINATION OF ANTIBODY AND ANTIGEN INTERACTION

- First spot appeared reddish purple just like gold conjugated antibody color (Fig. 3.1).
- Second spot appeared as circular appearance of dual lining with light green color in center and light reddish purple color on periphery (Fig. 3.1).
- The appearance of 3rd spot was astonishing as where a secondary antibody goat-anti-rabbit antibody (IgG) was immobilized and a mixture of conjugated solution (pH 8.8) and Bt positive extract(Cry1Ac-Antigen) was applied, the spot of IgG repelled that mixture and gave two overlapping circles of dual margins. The external peripheral margin of one circle was more overlapping with external peripheral of other spot and smaller with central margin of that (Fig. 3.1).
- The 4th spot Appeared in two partial circles of dual margins; central margin was smaller interacting with antibody spot (Fig. 3.1).

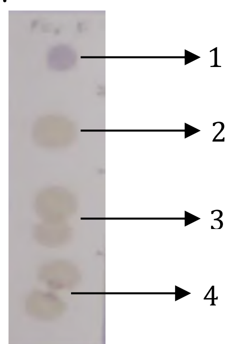

Fig 3.1 Determination of antibody–antigen interaction. 1: antibody gold conjugate solution, 2: conjugate solution and antigen mixture, 3: IgG, conjugate solution and antigen mixture, 4: Cry1AC antibody, conjugate solution and antigen mixture.

3.2 IMMUNO- BLOT FOR THE DETECTION OF ANTIBODY AND ANTIGEN INTERACTION BY USING 1X PBS AS PROTEIN ANTIGEN EXTRACTION BUFFER

This experiment was scheduled to interpret the role of protein extraction buffer in antibody–antigen reaction. So 1X PBS was used as protein extraction buffer (Fig 3.2) and results were the followings:

- A light green colored spot appeared when Bt negative sample and gold conjugate antibody was used.
- A light reddish circle of double margin appeared by using Bt positive sample and antibody-gold conjugate.
- On 3rd spot goat-anti-rabbit antibody (IgG) was spotted, dried on 37º for half an hour. A mixture of antibody-gold conjugate and Bt positive extract in 1X PBS was dropped. The mixture interacted with the antibody spot and gave dark reddish purple colored circle.
- On 4th spot Anti-Bt Cry1Ac antibody was dropped and dried on 37ºC for 30 minutes. A mixture of gold conjugate antibody and Bt positive extract in 1X PBS was dropped, the mixture reacted with antibody spot and dark reddish purple colored circle like shape appeared.

Both the spots of antibodies (Anti-Bt Cry1Ac antibody and goat-anti-rabbit antibody) appeared almost similar., which showed the antibody-antigen interaction.

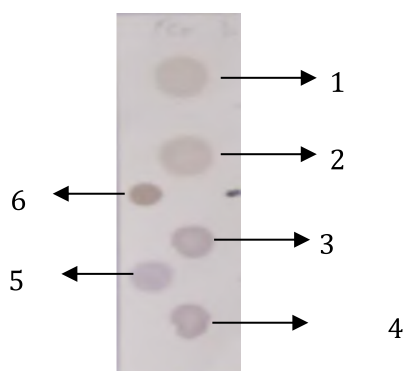

Fig 3.2 Blot test for the determination of antibody-antigen reaction by using 1X PBS as protein extraction buffer. 1: Bt negative sample and antibody-gold conjugate, 2: Bt positive sample and antibody-gold conjugate,3: Goat-anti-rabbit antibody (IgG), Bt positive sample and antibody-gold conjugate, 4: Cry1Ac-antibody, Bt positive sample and antibody-gold conjugate, 5: only antibody labeled gold, 6: nano-colloidal gold without antibodies.

3.5 VERIFICATION OF ANTIBODY ANTIGEN INTERACTION

- First spot with goat-anti-rabbit antibody (IgG) was dried at 37ºC for half an hour, Bt positive sample extract in 1XPBS and antibody conjugated gold gave a light reddish purple colour spot. When mixture of positive sample extract in 1X PBS and antibody conjugated gold was dropped on antibody spot, the interaction took place between antibody and antigen (fig 3.3).
- Second spot with goat-anti-rabbit antibody (IgG) was dried at 37ºC for half an hour, Bt negative sample protein extract in 1XPBS and antibody conjugated gold gave dark purple brown color (fig 3.3).
- 3rd spot with anti-Bt Cry1Ac antibody dried at 37ºC for half an hour, Bt positive sample extract in 1XPBS and antibody conjugated gold gave a light reddish purple color spot. When mixture of positive sample extract in 1XPBS and antibody conjugated gold was dropped on antibody spot, the interaction took place between antibody and antigen (fig 3.3).
- 4th spot with anti-Bt Cry1Ac antibody dried at 37ºC for half an hour, Bt negative sample extract in 1XPBS and antibody conjugated gold gave dark purple brown color.

The results were similar for both antibodies with positive and negative samples (fig 3.3).

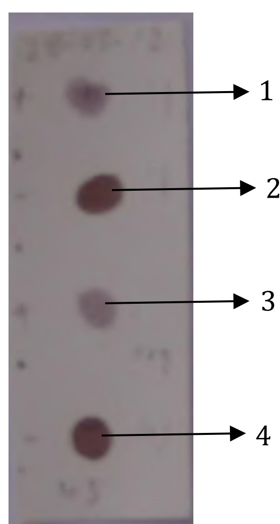

Fig 3.3 Verification of anti-Cry1Ac antibody and Cry1Ac antigen interaction; 1: Goat-anti-rabbit antibody (IgG),Bt positive sample extract in 1XPBS and antibody conjugated gold,.2: Goat-anti-rabbit antibody (IgG), Bt negative sample extract in 1XPBS and antibody conjugated gold, 3: Anti-Cry1Ac antibody, Bt positive sample extract in 1XPBS and antibody conjugated gold, 4: Anti-Cry1Ac antibody, Bt negative sample extract in 1XPBS and antibody conjugated gold.

4. DISCUSSION:

In order to attain quality results and stable antibody coated nano gold particles, several parameters should be measured[7]. These include: prediction of isoelectric point (pI) of the antibodies, the optimum pH of the reaction, the concentration of antibody loaded onto the nano gold particles, and the stability[8]. Generally, most antibodies can be adsorbed onto the metal surface in an optimal approach using buffers close to their pI value, away from this pH the adsorption decreases [9]. Gold nano particles were used in present study as color producing probes because these are reported to be inert, non-toxic, and have long stay of their optical properties, which makes them as a best choice for signal generation. The accumulation of gold nanoparticles produces a characteristic red color as a result of surface plasmon resonance [10]. Macromolecular ligands adsorb onto colloidal gold through a combination of electrostatic and hydrophobic interactions. Cry1Ac antibodies were conjugated with gold particles after adjusting the pH of colloidal gold solution. gold with a suitable amount of adsorbed Cry1Ac antibodies are not subject to flocculation with high salt, making this an excellent tool for ensuring whether the colloid has been sufficiently ‘‘protected’’[11]. After preparation of Cry1Ac antibody gold conjugate solution and to check stability of conjugation reaction; 10 µl of coated gold solution was mixed with 10 µl 1M NaCl[12]. The results under the optimized condition indicated color stability, which indicated that Cry1Ac antibody- nano gold particles are stable enough to be utilized in the immuno-blot experiments.

During conjugation colloidal gold and Cry1Ac antibody , it is important to control the pH of the Cry1Ac antibody and colloidal gold. Both measures should be adjusted to a pH slightly above the isoelectric point (pI) of the Cry1Ac antibody before conjugation[6]. Below the pI of the Cry1Ac antibody, Cry1Ac antibody induced flocculation may occur, whereas, above the pI of the Cry1Ac antibody, there is limited adsorption due to charge repulsion between the Cry1Ac antibody and the nano gold particles[13]. In experiments, which were designed for conjugation of nano gold and Cry1Ac antibody, 20 nm gold was used with OD 15. finalized pH values for conjugation process were 8.8 and 9.2, which were close to calculated pI value of Cry1Ac antibody i.e. pH 8.8.

The conjugation of antibody with colloidal gold is controlled by concentration used for full surface saturation of nano colloidal gold particles, which enhance the chance of antigen-antibody interaction after collision with antigen and also increased the stability by protecting the surfaces of colloidal gold particles against coagulation[14, 15]. The optimized concentration of Cry1Ac antibody for conjugation with nano colloidal gold was 14 µl of 2 mg/ml antibody solution in 1X PBS. After addition of optimized concentration of antibody to the colloidal gold solution, BSA blocking stabilizer buffer was added with the concentration of 50µl/0.5ml[16].

In immune assays, the primary function of a protein spotted to a nitrocellulose membrane is to act as a capture reagent for the target antigen in a sample [7]. In the present research work, Cry1Ac antibody was applied on membrane as a capture reagent and target reagent was Bt Cry1Ac protein extracted from Bt cotton seeds or leaves. Since the test result is totally dependent on achieving a good binding of the capture reagent to the membrane, therefore, achieving a high and consistent level of protein binding is very important[17].

During the antigen Bt Cry1Ac protein clarification and extraction, the antigen under examination can easily become unfolded or denatured, before clear identification. Proteins may also separate from the assay solution or become entangled with irrelevant cellular material like lipids and DNA[18].

An appropriate buffer solution provided to a protein mixture during the antigenic protein extraction process can improve the stability of protein to isolate them for study[19]. A buffer solution can protect the identity of the proteins while isolating them from other cell components[8].

To accomplish this goal, we had to choose a buffer solution that is compatible with the protein in question and creates an ionic environment as to the ionic environment of the cell.

5. CONCLUSION

The process described above is versatile and unique. it can be applicable to such diverse problems as the detection of recombinant proteins in plants and animals, and antigen detection in serum. Example is given here. Another basic advantage is that the use of nitrocellulose membrane allows the reaction to be visualised against a background which is almost white. In this way, the discriminatory power is greater than in a microtiter plate, and it becomes much comfortable to detect positive reactions and to reject false positives

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