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A REVIEW ON STATUS AND DETECTION OF TRANSGENIC CROPS/PRODUCE

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Abstract

The adoption and cultivation of transgenic crops is constantly increasing year by year globally including India despite several controversies and concerns. The regulatory authorities of different countries have set the mandatory/voluntary labelling requirements for these transgenic produce and made strict regulatory procedures for the approval to grow, import or utilize these transgenic crops as food or food ingredients. The different screening kits based on either transgenic DNA or proteins are being used which are much helpful in fulfilling these regulatory compliances on transgenic labeling. The present article focuses on different screening methods and significant current advances in such detection technologies.

Keywords: Transgenic Crops, PCR, Dipstick, ELISA, Immuno-PCR.

Introduction

The transgenic crops have been introduced into the market as environmentally safe pest control alternative to fulfill the increasing demand of food. The cultivation of transgenic crops has been increased continuously due to the economic benefits (CERA, 2012). The farmers benefited enormously from reduction in the number of insecticide applications, thereby reducing exposure for farmer to such harmful insecticides, and contributed to a more sustainable environment and better quality of life (James, 2014). Though the extent of production and cultivation of transgenic crops is increasing, the biosafety of these crops has been a matter of serious debate in recent years. This led regulatory authorities to implement a set of procedures for the approval as food ingredients worldwide and implemented appropriate mandatory/voluntary

labeling thresholds for transgenic produce (Viljoen, 2005; Marmiroli et al., 2008). These necessitated developing specific test kits to discriminate the transgenic materials which are mixed intentionally or unintentionally. The screening of transgenic crops is now important from safety point of view and various detection techniques are being used successfully.

Status of transgenic crops

A transgenic crop is a plant into which foreign gene(s) has been artificially inserted which produce novel proteins that confer tolerance against pests, herbicides, stress, etc (Engel, Frenzl & Miller, 2002). A typical transgene construct is composed of at least three elements such as promoter element, transgene, and terminator element (**Figure 1**). Cauliflower mosaic virus promoter (Pe35S) is frequently being used to drive the expression of transgenes in transgenic crops (CERA, 2012). The T-Nos terminator from Ti plasmid of *Agrobacterium tumefaciens* is the most common terminator and functions as a stop signal when reading the inserted transgene. In general, the transgenes used for insertion such as *cry1Ac, cry1Ab, cry2Ab, vip3A, cp4epsps, etc.* encode a specifically selected traits e.g., insect-resistant, herbicide-resistant, stress-tolerant, etc. A marker gene (e.g. *nptI, nptII, hpt, uidA*, *luc)* is also inserted in crop with its respective promoter and terminator to monitor the successful plant transformation (Chawla, 2002), and the plant transformation is carried out either by Gene-gun or *Agrobacterium* infection method (Allen, Spiken & Thompson, 2005; Gelvin, 2005).

The adoption and cultivation of commercialized transgenic crops is continuously increasing year by year. In 2014, the global area of transgenic crops has reached to 181.5 million hectares spread across 28 countries (James, 2014). The USA continues to be the lead producer of transgenic crops globally with 73.1 million hectares. Till date, several transgenic crops have been commercialized and entered into the global market such as soybean, maize, cotton, canola, papaya, alfalfa, squash, sugar beet, tomato, potato, wheat and brinjal (CERA, 2012). The transgenic soybean was continued to be the principal transgenic crop cultivated in 2014. In India only Bt cotton crop is allowed for the commercial cultivation in 2002 and cultivated 11.6 million hectares of Bt cotton in 2014 (James, 2014).

Screening kits for transgenic crops

The commercial entry of various transgenic crops/produce in the market has generated a need for suitable analytical methods to screen the transgenic produce and to fulfill the requirement of

regulatory compliance. The screening of transgenic crops has also become necessary tools to allow consumers to make an informed-choice. The detection and quantification of transgenic materials relies, either on transgenic DNA or transgenic protein(s) produced in transgenic samples. The DNA-based methods includes polymerase chain reaction (PCR), southern blot, real-time PCR, microarray and biosensors whereas protein-based methods includes dipstick strip, enzyme linked immunosorbent assay (ELISA) and immuno-PCR.

DNA-based kits

As DNA has much higher stability over protein, therefore, it is preferred target for detection of raw ingredients as well as processed materials. The DNA-based screening includes PCR, southern blot, microarray, and biosensors. Only PCR method has found broad application in transgenic screening as a generally accepted method for regulatory purpose.

1. Qualitative PCR method

PCR is a highly sensitive screening method and even detection of single molecule of DNA is possible. Therefore, material in very small quantity would be enough to identify the transgene. The PCR based method is a valuable tool for transgenic crops detection and traceability, since they allow a rapid screening of large number of samples. The generic markers representing genetic control elements are being used for routine PCR-based screening purposes such as 35S promoter (P-35S) (Akiyama et al., 2009; Holden, Levine, Scholdberg, Haynes & Jenkins, 2010), *npt-II* marker (Randhawa, Chhabra & Singh, 2009) and T-Nos3' terminator (Akiyama et al., 2009) as these elements are present in majority of transgenic samples.

The PCR products are distinguished by variable size in agarose gel electrophoresis. Gel allows the separation of amplified products based on fragment size and visualization in the gel matrix using dyes such as ethidium bromide. PCR targeting single template includes construct-specific PCR, event-specific PCR and gene-specific PCR (**Figure 1**). The screening of transgenic produce by singlex PCR has been widely reported for various transgenes e.g., *cry1Ab, cry2Ab, cry1Ac, vip3A, cp4-epsps* (Singh, Ojha & Kachru, 2007; Singh, Ojha, Bhatanagar & Kachru, 2008; Wu, Wu, Xiao & Lu, 2008; Ballari, Martin & Gowda, 2013).

The advancement over single target screening is the use of multiplex PCR which includes several primer pairs to permit the simultaneous detection of

several target sequences in one reaction (Ballari et al., 2013; Randhawa, Chhabra & Singh, 2010). Since the number of authorized transgenic events and their cultivated areas are rapidly increasing, the need arises to accelerate the quick transgenic detection with less effort. Further, the classical agarose electrophoretic techniques are being replaced by capillary gel electrophoresis (CGE), which allows rapid separation and automation. The combination of PCR and CGE was developed for simultaneous detection of multiple DNA targets with specialty of higher resolutions and shorter assay time (Nadal, Esteve & Pla, 2009; Garcia-Canas, Mondello & Cifuentes, 2010).

2. Quantitative PCR method

To fulfill the requirement of mandatory labeling above the threshold value of transgenic content, the development of quantitative detection system is required. The quantitative real-time PCR (qRT-PCR) represents the most powerful and sensitive technique for routine quantification of transgenic ingredients. The qRT-PCR allows continuous monitoring of the amplification products using the strength of fluorescence signal (**Figure 2**). The non-requirement of post-PCR analysis rendered the qRT- PCR as the most widely used approach in screening of transgenic produce. The quantification is carried out in the exponential phase of PCR, by comparing the number of amplification cycles required to reach a preset threshold fluorescence signal. The obtained fluorescence signal in the assay may use either non-specific intercalating dyes (SYBR Green I) or specific probes such as hybridization probes (molecular beacon), hydrolysis probes (TaqMan, CPT, LNA, and MGB) and primer-based technologies (AmpliFluor, Plexor, Lux primers). To assess the quantity of transgenic content in a sample, two parallel reactions, each containing the same amounts of template DNA is performed in which one targets endogenous reference sequence and other, the transgene-specific sequence. Relative quantification is achieved by comparing threshold cycle of the two amplified sequences (ÄCt method) and absolute quantification determined by comparison to the standard curve.

The RT-PCR utilizing SYBR Green I fluorescence dye is the simplest and cost-effective technique and employed successfully for screening and quantification of a range of transgenic samples (Terry et al., 2002; Andersen, Holst-Jensen, Berdal, Thorstensen & Tengs, 2006). Furthermore, the specific probes are currently being used for more specific detection of various transgenes in transgenic soybean, cotton samples such as TaqMan (Terry et al., 2002; Andersen et al., 2006; Yang, Guo, Zhang, Liu &

Zhang, 2008; Mano et al., 2009; Oguchi et al., 2009; Randhawa et al., 2010; Bahrdt, Krech, Wurz & Wulff, 2010), MGB TaqMan probe (Andersen et al., 2006), molecular beacons (Andersen et al., 2006), scorpion (Terry et al., 2002), LNA (locked nucleic acid) probe (Salvi, D'Orso & Morelli, 2008). Among them, TaqMan probes and SYBR green I are the most widely used RT-PCR chemistries. Furthermore, multiplex TaqMan based RT-PCRs are being targeted to reduce cost and time of the assay (Oguchi et al., 2009; Bahrdt et al., 2010).

3. Microarrays

DNA microarrays or "DNA chips" is an emerging DNA-based analytical method consist of oligonucleotide probes immobilized on a glass support (**Figure 3**). This approach enables the parallel multi-target detection of several PCR products in a single assay. These probes are specific for screening elements, species reference elements, and control elements. This method relies on direct hybridization of total genomic DNA in microarrays having designed probes with a set of reference sequences. Several microarray-based assays combined with multiplex PCR have been reported for detection of transgenic maize, canola, cotton, tomato and soybean events (Leimanis et al., 2006; Xu et al., 2007; Schmidt et al., 2008; Zhou, Zhang, You & Wu, 2008; Kim, Kim, Lee, Kim & Kim, 2010; Feng, Liang, Wang & Chen, 2013) by using fluorescent probes. DNA-based microarrays have the highest number of multiplexing capabilities and so far maximum simultaneous detection of nineteen transgenic events has been reported (Kim et al., 2010). Recently, a novel multiplex quantitative DNA-based target amplification method, named NASBA implemented microarray analysis (NAIMA) has been developed which is suitable for sensitive, specific and quantitative detection of transgenic materials on a microarray (Dobnik, Morisset & Gruden, 2010). Although the microarray analytical approach is relatively expensive, but it is one of the most promising reliable screening method at present owing to its flexibility and high-throughput capability, and proved to be a new method for routine analysis.

4. Biosensors

DNA biosensors are cutting-edge technology that focuses on rapid and inexpensive way of testing and represents an interesting alternative in transgenic screening (Sassolas, Leca-Bouvier & Blum, 2008; Arugula, Zhang & Simonian, 2014). It is based on hybridization reaction between an immobilised DNA probe attached to the surface of the sensor and a target DNA,

consisting of a probe complementary sequence (**Figure 4**). Recognition between the probe and DNA target generates a signal which is transmitted through a transducers (electrochemical, optical, and piezoelectric) and the signal is detected, measured, and processed to provide both qualitative and quantitative information. DNA biosensors may be based on optical (Kalogianni, Koraki, Christopoulos & Ioannou, 2006), electrochemical (Ahmed et al., 2009) and piezoelectric transducers (Passamano & Pighini, 2006). The biosensor is suitable for screening of raw ingredients as well as processed food products.

Protein-based kits

Protein-based kits are primarily based on immunoassays i.e. specific binding between an antigen and an antibody. This method includes enzyme linked immunosorbant assay (ELISA), immuno-PCR, dipstick strip assay, dot immuno-binding assay, western blot and HPLC/Peptide profiling. ELISA and dipstick are typical protein-based tests that have been generally used to detect the expressed transgenic proteins in plants. The protein-based kits are not suitable for detecting the expressed transgenic proteins in processed foods because the protein gets degraded in the processed foods/produce.

1. **ELISA test**

In general, two types of ELISA are being followed to measure the antigens. However, sandwich type of assay is used for screening and quantification of transgenic proteins between two layers of antibodies (**Figure 5**). ELISA combines the specificity of antibodies with the sensitivity of enzyme assays, by using antibodies coupled to an easily assayed enzyme. In this assay, one antibody (capture antibody) is purified and bound to a solid phase typically attached to the bottom of well. The protein is then added and allowed to combine with the bound antibody. The unbound products are then removed with a wash, and enzyme-labeled detection antibody (generally labeled with HRP) is allowed to bind to the protein, thus completing the "sandwich". The assay is then quantitated by measuring the amount of labeled second antibody bound to protein through the use of a colorimetric substrate. The sensitivity of ELISA depends on the colorimetric or fluorometric reaction between substrate and enzyme in final step of assay which can be visualised and measured when the protein and specific antibody bind together.

Currently, ELISA test has become important tool for routine analysis of expression levels of transgenic proteins.

Several ELISA tests have been developed for the detection and quantification of different transgenic proteins in various transgenic samples (Fantozzi et al., 2007; Roda et al., 2006; Ermolli et al., 2006; Paul, Steinke & Meyer, 2008; Shan, Embrey & Schafer, 2007; Wang et al., 2007). The ELISA kits are commercially available for detection of several transgenic proteins in transgenic crops (Agdia; Eurofins GeneScan; EnviroLogix; Romer Labs) and sensitivities of the ELISA assay have been reported upto pg/ml level (EnviroLogix).

2. Dipstick or immuno-chromatographic (IC) strip test

The dipstick assay uses a membrane-based detection system. It is a variant of ELISA, using nitrocellulose (NC) strips rather than microtiter wells. The dipstick includes a plastic backing card, NC membrane (5 – 15 μ pore size), sample pad, absorbent pad and conjugate pad (**Figure 6**). The NC membrane contains immobilized antibody on test line which is specific for the target protein. For the visual detection of results, the conjugates preparation is required which acts as a probe and provides the color development over the assigned lines. The conjugates are prepared by combining the anti-antigen antibody with labeling agent. Several choices of labeling agents have been used for the development of strip such as gold, carbon, selenium, up-converting phosphor (UCP), magnetic nanoparticles (Huang, Aguilar, Xu, Lai & Xiong, 2016), and enzymes (Maiolini et al., 2014). However, colloidal gold is used as label in most of the membrane based rapid tests owing to their greater stability, sensitivity and precision which make it suitable for use in paper-based tests over other labeling agents (Chandler, Gurmin & Robinson, 2000).

The NC membrane contains two captured zones, one specific for the transgenic protein and another specific for untreated antibodies coupled to the color reagent. The conjugate pad containing immobilised labelled antibody specific for protein is separately placed on the strip to respective position. When strip is placed in a vial containing an extract from plant tissue or seed harbouring transgenic protein, samples moves in upward by capillary action and develops color on the captured zones. The dipstick format gives qualitative results either presence or absence of protein in transgenic sample within a few minutes. The sandwich format of dipstick is generally used for screening. In this format positive results can be visualized by formation of two red colored lines on the membrane (**Figure 7**).

Screening of the results is determined visually providing quick qualitative answer in "yes" or "no" and employed successfully for detection of various transgenic proteins (Kumar et al., 2010; Kumar, 2012a). This paper-based method provides affordable, user-friendly, rapid, robust screening of samples. Thus, dipstick has a great potential to deliver point-of-care detection in a resource-limited set-up. Dipsticks are also commercially available to detect several transgenic proteins such as Vip, Cry1Ac, Cry1Ab, Cry2Ab, CP4-EPSPS, etc. in different transgenic crops/ produce (Agdia; EnviroLogix; Romer Labs). Furthermore, several commercial strips have been developed for simultaneous detection of two or more transgenic proteins, such as Cry1Ac, CP4EPSPS, and Cry2A/Cry1F to reduce cost and time of assay (Agdia; Eurofins GeneScan; EnviroLogix; Romer Labs). Several advancements in the dipstick have been carried out such as multianalytes detection in a single assay (Kumar, 2012a), improvement of sensitivity using enhancer (Kuang et al., 2013) and quantitative readout (Byrnes, Thiessen & Fu, 2013: Hu et al., 2014). Simultaneous detection of antigens in the target samples can be obtained without using expensive and complex instrumentation which thereby minimize the cost of analysis as well as duration of assay.

3. Immuno-PCR test

The IPCR is very much similar to ELISA except that the detection antibody that is linked to the short reporter DNA sequences instead of a conventional enzyme which further is amplified either by conventional PCR or by real-time PCR (**Figure 8**). It combines the advantages of specificity of immunoassays and sensitivity of PCR. In conventional IPCR the amplified reported DNA can be analysed by agarose gel electrophoresis but sometimes the non-specific results would also be observed. To minimise this non-specific results, RT-IPCR would be employed which involves the RT-PCR technology. RT-IPCR assay has been extensively used in field of immunological research and clinical diagnostics to increase the sensitivity of detection where complex biological matrices and small amounts of sample available. Due to some limitations such as high cost, time consuming, and requirement of expensive instruments, this technique is not routinely utilized for transgenic detection. Still we find some progress in transgenic detection and quantification Cry1Ac protein (Allen, Rogelj, Cordova & Kieft, 2006), Cry1Ab protein (Kumar, 2012b) and Vip3A protein (Kumar, 2011) owing to high sensitivity and requirement of small amounts of protein over other protein-based methods.

Conclusion

In spite of several controversies over transgenic crops, its adaptation is continuously increasing. Keeping in the view of specific labeling legislations over release of transgenic products, the development of specific and sensitive screening kits have become necessary to screen commercially approved transgenic products. Although a diverse screening kits and strategies have been developed and commercially available to fulfil the demanding issues of labelling regulations and screening of their produce. Among them, PCR, RT-PCR, ELISA, and dipstick are being widely used for such purposes. DNA-based kits targeting transgene in crops are generally being employed for highly sensitive and reliable results in a laboratory set-up utilizing complex instruments. However, protein-based kits detect and quantify the expressed transgenic protein thereby give the data on functional stability of the trangene inserted in target crops. These kits enable the high throughput screening of a large number of samples, and play a crucial role in the low-cost, rapid and routine screening systems for transgenic produce.

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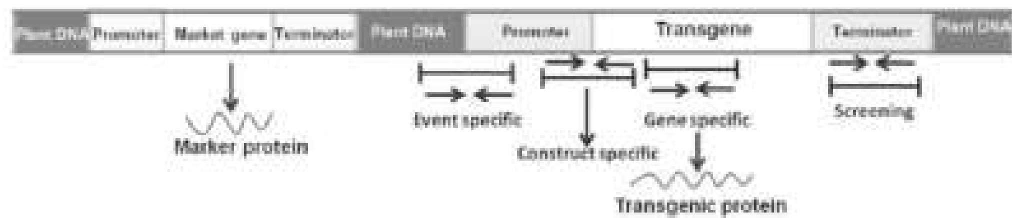

Figure 1. Schematic illustration of a typical transgene construct with marker gene. Arrows indicate the primer pairs targeting particular sequences around and within the transgene integration site. Four assay types illustrated targeting screening sequences (promoter P-35S, transgene, terminator T-Nos), sequences within the gene of interest; construct-specific junction sequences; and event-specific junction sequences.

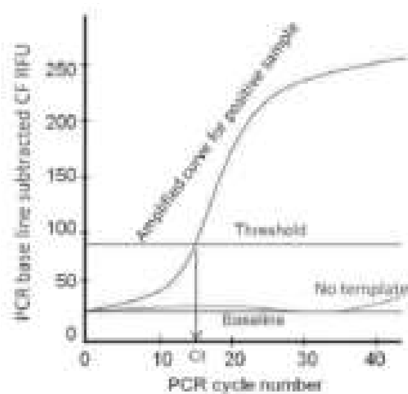

Figure 2. Schematic illustration for quantitative analysis of transgenic sample by RT-PCR. The transgenic contents are calculated by threshold cycle (Ct) value.

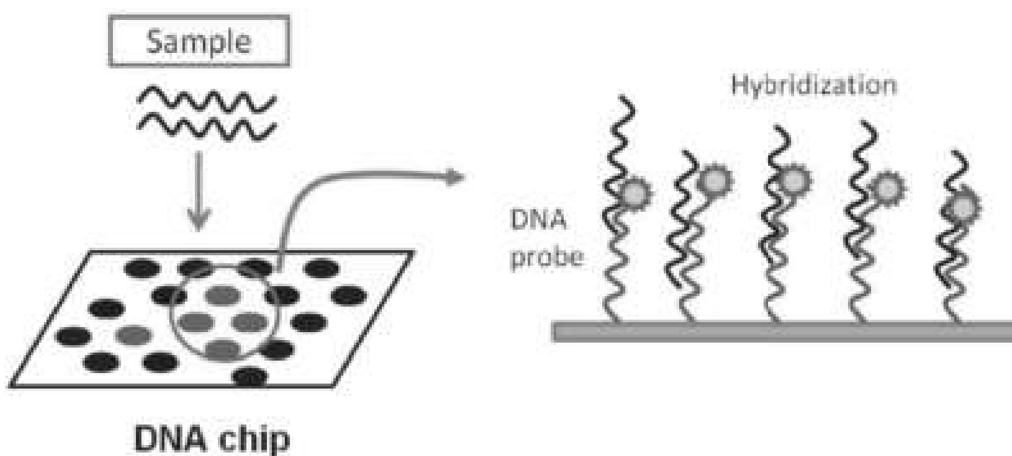

Figure 3. Schematic illustration of microarray-based screening.

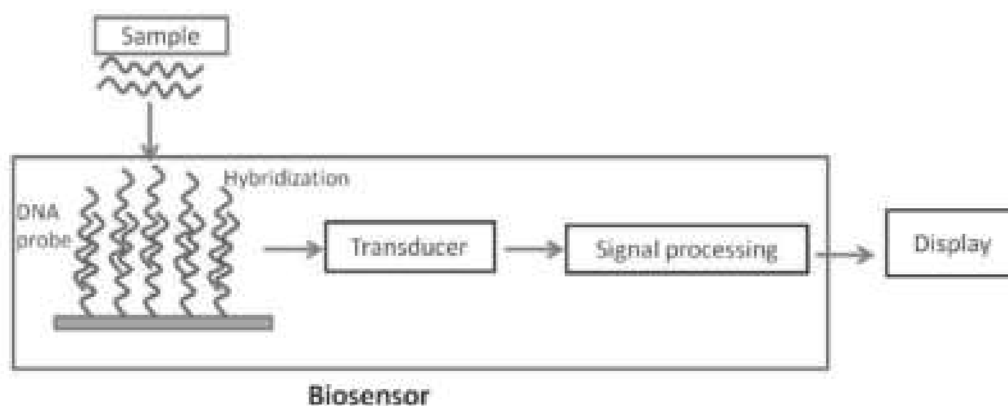

Figure 4. Schematic illustration of biosensor-based screening.

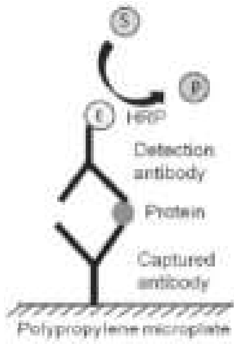

Figure 5. Sandwich ELISA using two antibodies i.e., captured antibody and detection antibody sandwiched transgenic protein. E, enzyme; S, substrate; P, product.

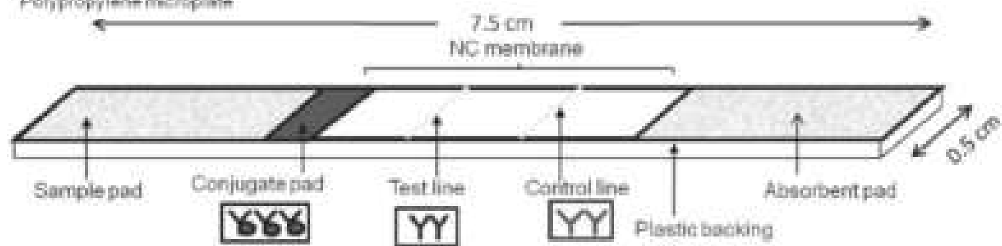

Figure 6. Schematic representation of typical dipstick strip. Anti-protein IgG is immobilized at the test line (T) and anti-species IgG is immobilized at the control line (C).

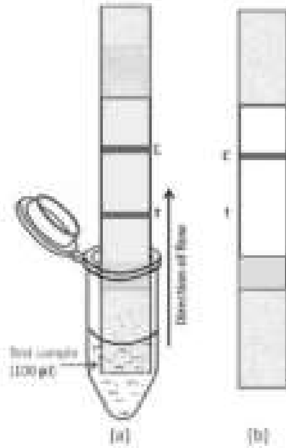

Figure 7. The illustration of dipstick assay results: **(a)** Sandwich format and the positive result is judged by the appearance of red line in test region; **(b)** Negative result, red line in control region should always visible showing valid test assay. T, test line; C, control line.

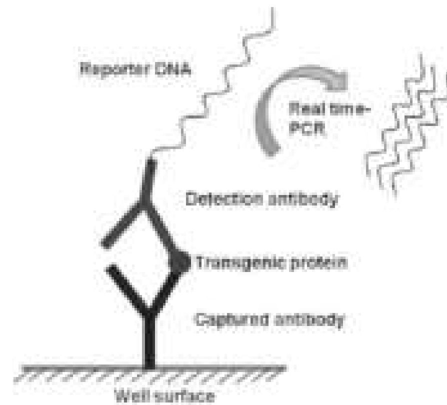

Figure 8. Sandwich type RT-IPCR assay