HLA-restricted and Antigen-specific CD8+ T Cell Responses by K562 Cells Expressing HLA-A*0201

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ABSTRACT

Background: Identification of antigen-specific T cells has yielded valuable information on pathologic process and the disease state. Assays for quantification of inflammatory cytokines or lytic-granule molecules have been generally used to evaluate antigen specific T cell response, however their applicability have been hampered due to the limited source of autologous antigen-presenting target cells (APC). **Methods:** K562, a leukemic cell line deficient of human leukocyte antigen (HLA), was transfected with a gene encoding HLA-A*02 (K562/A*02) and its function as stimulator cells in inducing activation of HLA-matched T cells was evaluated by IFN-γ enzyme linked immunospot (ELISPOT) assay. **Results:** The stable transfectant K562/A*02 pulsed with HLA- A*02 restricted peptide could specifically induce IFN-γ secretion by CD8+ T cells compared to no detectable secretion by CD4+ T cells. However, CD56+ NK cells secreted IFN-γ in both K562/A*02 with peptide and without peptide. The number of IFN-γ secreted CD8+ T cells was increased according to the ratio of T cells to K562 and peptide concentration. Formalin-fixed K562/A*02 showed similar antigen presenting function to live K562/A*02. Moreover, K562/A*02 could present antigenic-peptide to not only A*0201 restricted CD8+ T cells but also CD8+ T cells from A*0206 donor. **Conclusion:** These results suggest that K562/A*02 could be generally used as target having specificity and negligible background for measuring CD8+ T cell responses and selective use of K562 with responsder matched HLA molecules on its surface as APC may circumvent the limitation of providing HLA-matched autologous target cells. **(Immune Network 2006;6(4):179-184)**

Key Words: K562, HLA-A*0201, peptides, CD8+ T cells, IFN-γ ELISPOT assay

Introduction

Detection of viral and tumor antigen specific T cell response contributes to a continually increasing number of immunotherapy trials (1,2). In order to measure the quantity and quality of T cell response, target cells capable of presenting antigens in human leukocyte antigen (HLA) specific manner need to be provided. Although dendritic cell (DC), B cell, and macrophage are well known as professional antigen presenting cell, the use of such cells in immunological assays has been limited due to preparation of sufficient number of available autologous antigen presenting cells (APC). Particular emphasis is on time consumption and laborious steps required to generate DC from monocytes *in vitro* (3). Therefore, the use of a universal allogeneic antigen presenting cell line has received risen attention for its convenience.

Recently, several artificial antigen presenting cells have been investigated using insect cell (4,5), mouse fibroblasts (6), or human leukemic cell (7,8). One of these cells, K562 cells frequently used as targets cells for detection of cytotoxicity by natural killer (NK) cells have advantage avoiding alloreactivity due to the lack of HLA class-I and class-II (9-12). Moreover, since K562 cell naturally expresses adhesion molecules including LFA-3 and ICAM-1, there is no need to artificially to express such molecules.

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A variety of assays measuring the frequencies and functional properties of antigen-specific T lymphocytes has been established and its efficient use has been reported. The major analysis for antigen-specific T-cell response includes use of peptide-MHC tetramer (13,16) for antigen specific receptors, intracellular cytokine staining, cytokine secretion assay, and enzyme linked immunospot (ELISPOT) assay for quantitative measurement of cytokine production (14). The IFN-γ ELISPOT assay that allows the frequency of antigen-specific T lymphocytes to rapidly evaluate in single cell level has been applied by several groups to sensitively monitor the induction of antigen-reactive T cells in immunotherapy trials (15-17).

In this study, by performing ELISPOT assay, we demonstrate that K562 transfected with HLA-A*0201 gene can be used as efficient antigen presenting cell, which presents HLA restricted peptide to T cells and induces negligible background .

Materials and Methods

*Generation of K562 expressing HLA-A*0201 gene.* A stable transfectant K562/HLA-A*0201 was established by transfecting K562 cells with 5μg of the pcDNA3/HLA-A*0201 using Cell Line Nucleofector™ kit V, according to the manufacturer's instructions (Amaxa). After 24 h, expression level on the HLA-A*0201 cells was detected by FACSCalibur (Beckton Dickinson). Transfected cells were selected and subsequently cloned in selection medium containing G418 (1 mg/ml, Duchefa). To keep the expression, the transfectants were periodically sorted by FACS-vantage flow cytometer (Beckton Dickinson). K562 cells stably expressing single HLA-A*0201 alleles after transfection are referred to as K562/A*02.

The human CML cell line K562 and K562 expressing HLA-A*02 was maintained in RPMI 1640 medium (Cambrex) containing L-glutamine (GIBCO), antibiotics (Cambrex) and 10% FBS (Sigma). Cells were kept at a concentration of 1×10^5 to 10×10^5 cells/ml and cultured at 37°C in a humidified atmosphere supplemented with 5% CO_2.

Selection of CD8+ lymphocytes from PBMC. Nine healthy donors subjected in this study were CMV-seropositive. Their HLA types were determined by PCR-SSP and SBT. PBMCs were isolated from heparinized blood of donors by centrifugation on a Ficoll-Paque density gradient. Subsequently, CD8+, CD4+ and CD56+ cells were sequentially isolated with immunomagnetic beads following the manufacturer's instructions. For purification of magnetic enriched cells, we have used an Automated Magnetic Activated Cell Separation (autoMACS).

Antibodies and immunofluorescence analysis. The following monoclonal antibodies (mAbs) were used for immunofluorescence analysis; an anti-HLA class I, anti-HLA class II, anti-CD80, anti-CD86 and anti-CD54 Ab. Each surface molecule was analyzed by FACScalibur (Beckton Dickinson). Cells were washed twice with HBSS containing 2% FBS, were incubated for 15min at 4°C with each antibodies. After incubation, the cells were washed twice with identical HBSS buffer. FACS analysis was performed on FACScalibur (Beckton Dickinson).

Peptide. Peptides were used herein Human Cytomegalovirus (HCMV) pp65 peptide 495-503 (NLVPMVATV) and pp65 peptide 341-350 (QYDPVAALFF). HCMV pp65 peptide 495-503 was HLA-A*02 restricted, but pp65 peptide 341-350. All peptide was synthesized by Anygen (Gwangju TP, Korea). Peptides were dissolved in dimethylsulphoxide and then diluted in PBS.

*Peptide pulsing and fixation of K562/A*02.* Target cells pulsed with proper peptide were washed twice with serum free culture medium, RPMI 1640 (Cambrex). After centrifugation, supernatant was eliminated and the cells were resuspended 1% paraformaldehyde for 20 min in ice. Subsequently, cells were washed twice to three times with RPMI 1640. Fixed cells were kept at 4°C until use.

IFN-γ ELISPOT assay. For ELISPOT assays, 96-well nitrocellulose-bottom plates were coated with 100μl/well of anti-human IFN-γ at a 5μg/ml (BD Bioscience) and incubated at 4°C overnight. The following day, plates were washed once with RPMI containing 10% heat-inactivated FBS. After blocking with RPMI/10% FBS (2 h, RT), pre-incubated CD8+, CD4+ and CD56+ cells and K562/A*02 were seeded in duplicate. Control wells contained CD8+ T cells alone, CD8+ T cells in the presence of unloaded APC and APC alone. Culture medium was RPMI medium containing 10% FBS at a final volume of 100μl/well. Cells were incubated overnight (20～24 h) at 37°C in 5% CO_2 in a water-saturated atmosphere.

Plates were washed twice with deionized water and three times with PBS containing 0.05% Tween-20 (PBS/T). Captured IFN-γ was detected by incubation for 2 hr with 100 μl/well of biotinylated anti-IFN-γ at a concentration of 2μg/ml in PBS with 10% FBS at room temperature. After washing three times with 200μl/well PBS/T, streptavidin-HRP was added 100μl/well for 1 hr at room temperature. ELISPOT plate was washed four times in PBS/T and again twice in PBS. Plate was developed by AEC substrate and chromogen for 3～5 min and stopped by washing wells with deionized water. Spots were counted using an AID ELISPOT reader, and average total spots for duplicate wells were calculated.

Results

*Characteristics of K562/A*02.* Expression level of HLA-A*0201 gene of K562/A*02 cells was compared with

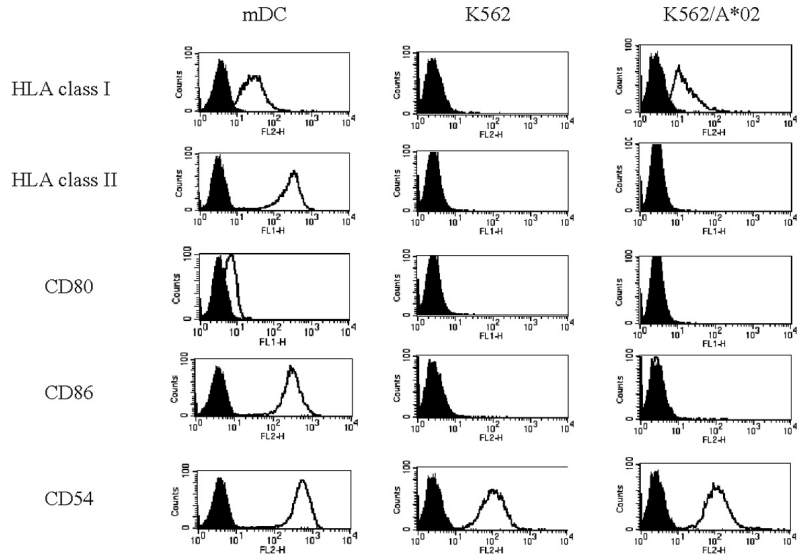

Figure 1. Flow cytometry analysis on K562/A*02, K562 and mDC. Establishment of stable transfected cell line expressing HLA-A*0201 gene depends on combination of a drug selection, G418, and fluorescence-activated cell sorting (FACS). Expression of HLA class I, HLA class II, CD80, CD86 and ICAM-1 (CD54) on K562/A*02 is compared with K562 and mDC.

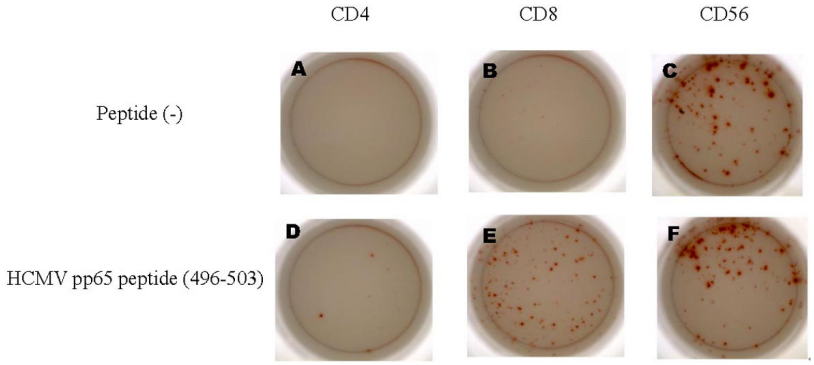

Figure 2. Recognition of antigen peptide-loaded K562/A*02 cells by CD8+ T cells. After PBMC isolated from healthy donor was separated to CD4+ and CD8+ T cell, ELISPOT assay was carried out. K562/A*02 cells were loaded with the HCMV pp65 A*02 496-503 peptides. For fresh PBMC (A～C), upper pictures are shown from ELISPOT wells containing responder cells with peptide-unloaded target. Lower panels are displayed ELISPOT plates containing identical responder cells with K562/A*02 holding the peptide. Each plate shows the number of IFN-γ spot-forming cells per 5×10^4 CD8+ T cells from the each well. All experiments yielded nearly identical results.

mDC and natural K562 (Fig. 1). Both mDC and K562/A*02 cell expressed HLA-A*0201, but the expression level was relatively decreased in K562/A*02 than that of mDC. However, mDC expressed costimulatory molecules, such as CD80 and CD86, and adhesion molecule like CD54, while K562/A*02 cells did not express CD80 and CD86, and expressed only CD54.

*Cells stimulated by K562/A*02 cells.* To examine whether established K562/A*02 cells are suitable stimulator to activate CD8+ T cells, CD4+, CD8+ and CD56+ cells from the peripheral blood of HLA-A*02 and HCMV-seropositive donors were purified by auto-MACS. Subsequently, the isolated cells were incubated with K562/A*02 cells and activation of the cell was monitored by IFN-γ ELISPOT assay. K562/A*02 cells expressing the HLA-A*0201 was only recognized by CD8+ T cells but CD4+ T cells (Fig. 2). As a control, T cells were incubated with antigen-free target cells. K562/A*02 pulsed with HCMV pp65 antigen peptide 495-503 efficiently stimulated CD8+ T cells; however, the only presence of HLA-A*02 molecule did not induce IFN-γ secretion when antigen was not loaded.

Both K562 and K562/A*02 cells expressing HLA molecule, cytotoxic activity by CD56+ NK cells was still triggered. This result suggests that not only K562 but also K562 expressing only single allele can still be targeted by NK cells, which can be attributed to the absence of NK inhibitory ligand on the surface.

*Factors influencing ELISPOT assay using K562/A*02.* To adjust the sensitivity of the ELISPOT assay, we incubated fixed numbers of CD8+ T cell with K562/A*02 cells at the ratio of 1 : 1 and 10 : 1. Although CD8+ T cell was activated at both ratios, Fig. 3A shows that the relevant proportion of stimulator to responder may be at 1 : 1. At the ratio 10 : 1, IFN-γ spot number was notably decreased. In addition, even if the ratio was 1 : 1, the number of responder was dropped to basal level when the number of responder cell was too small. We also tested the effect of various peptide concentrations on the activation of T cells. Fig. 3B shows that higher peptide concentration could stimulate more memory CD8+ T cells.

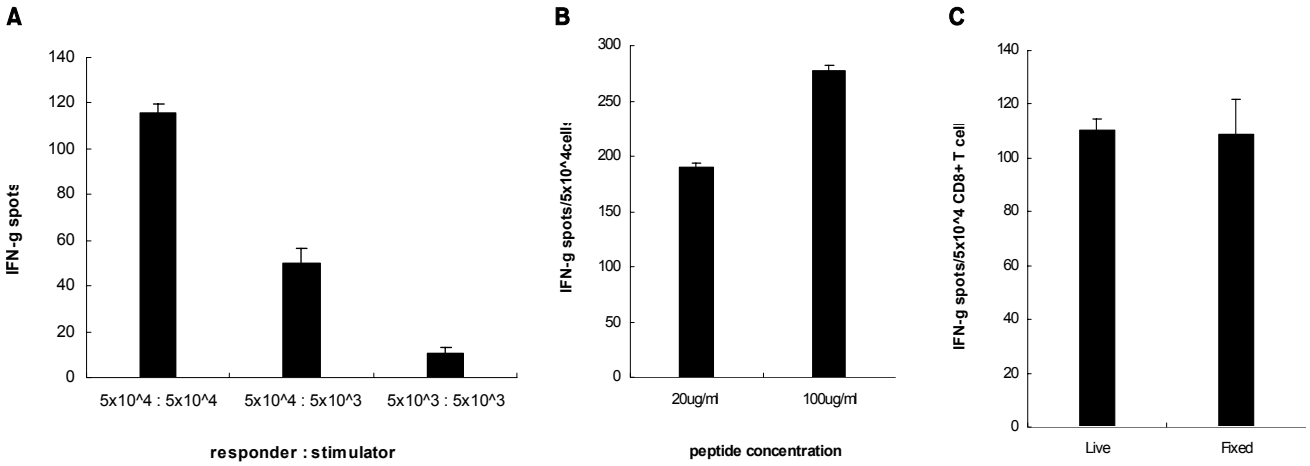

Figure 3. Determination of ELISPOT condition using K562/A*02 cell as target cell. (A) Comparison of a ratio of responder and stimulator cells. The ratio was 1 : 1 and 10 : 1. (B) Variation of ELISPOT spot number according to peptide concentration. Peptide concentration pulsed to the cells was applied for 20μg/ml and 100μg/ml. (C) Usage of fixed K562/A*02 by means of ELISPOT assay target. ELISPOT assay was carried into effect using K562/A*02 pulsed with peptide, which was in a state of live or fixed by 1% paraformaldehyde. Each experiment was performed in duplicate; the standard deviation is shown.

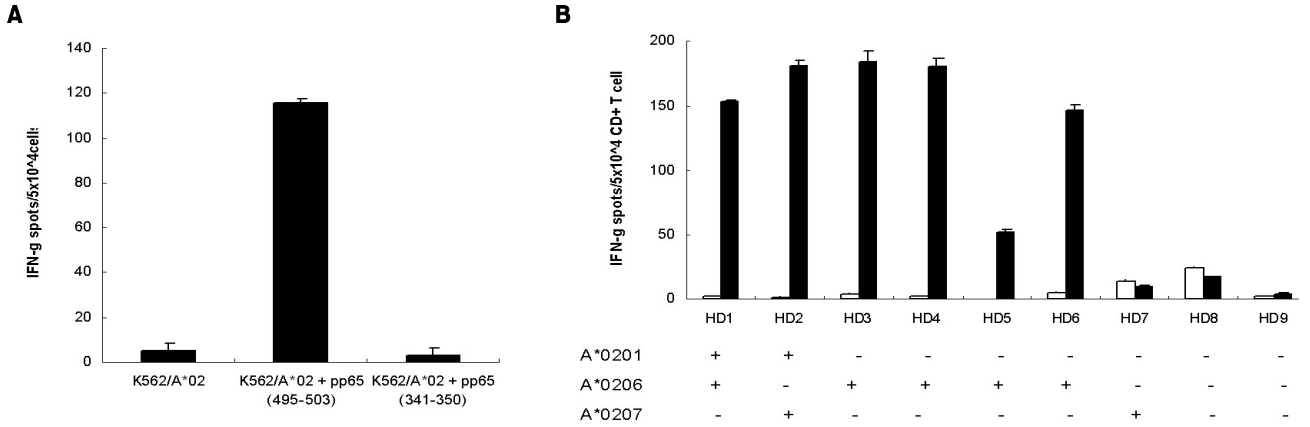

Figure 4. Application of K562/A*02 cells as target cell on the activation of HLA-A*02-restricted HCMV memory CD8+ T cells. (A) The CD8+ T and K562/A*02 cells pulsed with HCMV pp65 495-503 peptide were seeded at 5×10^5 cells per well. As controls, CD8+ T cell were incubated with unpulsed K562/A*02 or K562/A*02 cells with irrelevant peptide. (B) CD8+ T cells were purified from PBMC of 9 healthy donors (HD1～9). HD1 to HD7 was positive for HLA-A*02 whereas HD8 and HD9 was not. All healthy donors were CMV-seropositive. CD8+ T cells were incubated with K562/A*02 cells loaded with pp65 A*02 peptide (black bar). As controls, the T cells were incubated with antigen-unpulsed K562/A*02 cells (white bar). Each column depicts the average of all experiments, n=2, and error bars represent standard errors.

From these results, because K562/A*02 loaded with 100μg/ml peptide generated too many spots to count, peptide concentration of 20μg/ml was used in this study.

To use the target cell more efficiently, we also used target cell fixed in 1% paraformaldehyde during ELISPOT assay (Fig. 3C). When compared between live and fixed K562/A*02 as target cell in this assay, no significant difference in frequency of CD8+ T cells activated by both target cells was found. When the fixed cells stored for 2 weeks at 4°C were used as target, the IFN-γ secretion induced by the fixed target was similar to that of the live target (data not shown).

*HLA-restricted antigen presentation by K562/A*02 cells.* To investigate whether K562/A*02 was restricted in HLA-A*0201 CD8+ T cell, the cells were loaded with HCMV pp65 A*02-binding peptide (495-503), HCMV pp65 A*24-binding peptide (341-350). The CD8+ T cell from HLA-A*02 positive and HCMV positive healthy donor was used as responders. As shown in Fig. 4A, the IFN-γ spot number induced by CD8+ T cells activated with K562/A*02 pulsed with HCMV pp65 A*02-binding peptide was significantly increased compared to those of CD8+ T cells incubated with K562/A*02 without antigen peptide or irrelevant pep-

tide, pp65 A*24 peptide. K562/A*02 did not induce CD8+ T cell activation when loaded with irrelevant peptide or not loaded with any peptide.

We further investigated the ability of K562/A*02 transfectant to present peptide antigen to T lymphocytes bearing not only HLA-A*0201 but also other A*02 subtype. The assay was carried out with several A*02 subtypes bearing T cell as responder from A*02 positive donors (Fig. 4B). HD1-2 possesses a HLA-A*0201 allele with different subtype alleles, A*0206 or A*0207. CD8+ T cells of the HD1-2 was efficiently activated by K562/A*02 with HCMV pp65 HLA-A*0201 restricted peptide. Also, CD8+ T cells from HD3 to HD6 bearing only HLA-A*0206 could be activated by K562/A*02 with pp65 peptide. However, CD8+ T cells from HD7 bearing only with HLA-A*0207 was not activated by K562/A*02, and produced low spot number as that of negative control, A*02 negative donor HD8 and HD9. These results suggest that K562/A*02 transfectant is specific not only to HLA-A*0201 type but also HLA-A*0206.

Discussion

Among the current assays measuring frequency and functional properties of antigen-specific T cells, IFN-γ ELISPOT assay has been a favorable choice because the assay requires very low number of responder and stimulator cells and is still able to detect IFN-γ secreting T cells when limited number of cells are provided (3,5). Despite of its superior sensitivity over the other immunological assays, a major limitation of assay measuring T cell response is to provide suitable target cells in a MHC-restricted manner (3), thus a various strategy establishing artificial APC that serves as an optimal artificial APC has been sought.

major advantage on the use of K562 over other cells is the deficiency of HLA molecules, which allows elimination of concerns for non-specific alloimmune response. In addition to that, K562 naturally expresses several adhesion molecules required to activate T cells. Moreover, the use of K562 transfectant as target cell demonstrated the lower background than other commonly used target cells including TAP-deficient (T2) cells, autologous DC or LCL in ELISPOT assay (3, 14,18-20). Next, we tested whether the presence of single allele, HLA-A*0201, can avoid cytotoxic activity of NK cells against K562 expressing HLA-A*0201. Despite of the matched HLA alleles, NK cells showed cytotoxicity against K562/A*02, which can be attributed to the absence of NK inhibitory ligand on the surface. This is consistent with the previous report that the inhibitory receptor of NK cell was activated by ligand for HLA-A*3, HLA-A*11, HLA-C*1, HLA-C*2, HLA-E and HLA-G (25). Thus PBMC including NK subsets are not appropriate to measure antigen-specific response through IFN-γ ELISPOT assay, but better to use purified CD8+ T cells for the assay.

pp65 is representative HCMV antigen. HCMV is a member of the Herpesvirus family that infects 60% to 90% of individuals, depending on the population (23). Thus, most of the HCMV-seropositive individuals contain memory T cell circulating peripheral blood against HCMV. From this, pp65 has been selected for antigen target, which allowed detection and quantification of low numbers of antigen-specific T cells in freshly isolated blood lymphocytes without the need for in vitro expansion (21). During the experiment, we expected that K562/A*02 could survive against cytotoxic activity by NK cells because these cells are characterized by the ability to kill cells that lack HLA molecules while sparing autologous normal (HLA positive) cells.

To verify the specificity of the transfectant, we also pulsed K562/A*02 cell with HLA-unmatched antigenic-peptide and cultured with identical CD8+ T cells. As expected, in contrast to K562/A*02 with relevant peptide, K562/A*02 with irrelevant peptide could not present the antigen to CD8+ T cell and thus could not stimulate T cell (Fig. 4A). Our results also show that K562/A*02 with HLA-A*02 specific peptide could efficiently stimulate not only HLA-A*0201 but also HLA-A*0206, but not HLA-A*0207. In a previous study about functional HLA supertype, Sette end Sidney established that simple epitope could be capable of binding multiple HLA molecules. The HLA-A*02 supertype is one of the most prevalent HLA supertypes that include, minimally, A*0201, A*0202, A*0203, A*0204, A*0205, A*0206, A*0207, A*6802, and A*6901 (22,24). A*0201 and A*0206 alleles have high-binding affinity to peptide, while A*0207 does not. In other words, A*0201 is possible to have cross-reactivity with A*0206, not with A*0207. Thus, heterozygote donor with A*0201/A*0206 and A*0201/A*0207, and haplotype donors with A*0206 showed positive response to K562/A*02 : A*0201 pp65 peptide complex. However, A*0207 donor revealed negative response to K562/ A*02 : A*0201 pp65 peptide complex (Fig. 4B).

In this study, we suggest that antigen-specific CD8+ T cells can be conveniently and specifically activated by artificial APC, K562/A*02, and the results of this study should be useful for the development of artificial APC-based immunoassay.

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