YAMAKIN

From Production Skills to Scientific Knowledge,
and onward to Fusion with Medical Science

Light and Heat-Cured Indirect Composite Resin

# Safety Evaluation Report

## Biological Evaluation of "TWiNY" Hybrid Composite Resin

Vol. 8 in a Series on Safety Reports


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Biological Science Safety Laboratory

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# 1. Introduction

Metals, ceramics, and resins are used for dental prosthesis; besides strength and durability, safety is an absolute requirement in the oral use environment. In particular, the use of resin material has become more and more widespread because of its various strengths in the clinical context, such as excellent workability and reproduction of natural teeth. Against this background, our company commenced laboratory studies on indirect composite resin; we have also published extensive research findings on filling ratios and the physical properties of inorganic fillers and monomers. Moreover, to further strengthen the safety aspect of our product range, we have carried out a range of tests concerning cell, cell tissue, and genetic engineering, in collaboration with the Dental Surgery Science Course at Kochi Medical School, Kochi University, Japan. In June, 2006, the development of “Luna-Wing”, indirect composite resin which is safe for use in the human body, reached fruition.

In today’s clinical environment, patient demand for implant treatment is expanding, and there is also increasing demand for prosthetic materials for implant superstructures which offer higher strength and better esthetics than indirect composite resin. We took Luna-Wing as a starting point for the development of a new type of indirect composite resin. Based on Luna-Wing, a new inorganic filler was successfully discovered through the laboratory studies on indirect composite resins; the research goal was to create higher strength and durability, so maintaining good workability while simultaneously taking the safety of the component materials into consideration. Subsequently, research on filling ratios and additives was repeatedly performed, leading to the development of TWiNY. TWiNY is a light and heat-cured indirect composite resin which exhibits higher fatigue strength and excellent durability compared to conventional indirect composite resin. It also has better workability, an abundant color lineup, and excellent esthetics. As a guiding development concept, our company takes safety as a major focus; we insist not only on “Excellent Physical Properties” in our products, but also on “Biological Safety” In this respect, the safety of TWiNY has been confirmed in *in vivo* examination to be in compliance with ISO 10993, “Biological Evaluation of Medical Devices”.

In order to ascertain the impact of TWiNY, light and heat-cured indirect composite resin in the oral context in more detail, biological safety evaluations using three methods of stain-exclusion test were undertaken in collaboration with the Dental Surgery Science Course at Kochi Medical School, Kochi University. These tests comprised: 1) PHK16 Cells (epithelial cell) Proliferation Test, assuming impact on the oral mucosal epithelium 2) V79-Cell (fibroblasts) Colony-Forming Assay, assuming impact on connective tissue and 3) THP.1 Cell (leukocyte) Trypan Blue Exclusion Test, assuming impact on the immune system. Compiling these test results here, we report them as “Light and Heat-Cured Indirect Composite Resin Safety Evaluation Report: Biological Evaluation of “TWiNY” (Vol. 8 in a Series on Safety Report)”. The information yielded through these experiments may be confidently expected to help ensure the security of both medical professionals and patients. Also, please refer our technical report for information on the basic physical properties and optical and technical properties of TWiNY.


Yamamoto Precious Metal Co., Ltd.
Managing Director/Doctor of Engineering:
Teruo ANRAKU

# 《 2. Materials and Methods 》

## 2.1 Materials

The light and heat-cured indirect composite resin TWiNY was used as a material and the indirect composite resin Luna-Wing was used as a comparative material. The materials were prepared as shown below. Each material was formed into a round shape using a metallic ring (15mm diameter, 1mm thickness) held between polyethylene terephthalate films .and light-cured on each side for 3 minutes. For the light and heat-cured composite resin materials, heat-curing was carried out for 15 minutes at 110°C after light-curing. A test piece was then made by polishing the surface of each test material with waterproof abrasive paper, at least 0.05mm, and buffing the material until it became glossy (Fig.1).

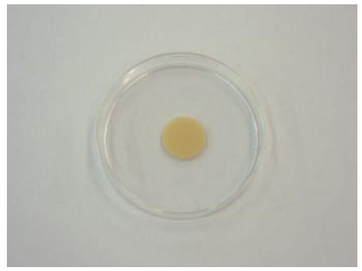


Fig.1: Specimen

## 2.2 Methods

In this evaluation, tissue is broadly classified into three types in order to simulate each of the relevant cells: primary human keratinocytes (PHK16 Cells: Fig.2), Chinese hamster fibroblast (V79 Cells: Fig. 3), and human monocytic leukemia (THP.1 Cells: Fig 4) were used as analogs for epithelium tissue, connective tissue and the immune system respectively to perform this test (Table 1) .

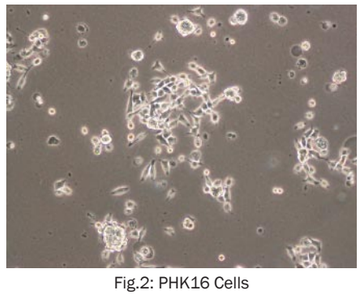


Fig.2: PHK16 Cells

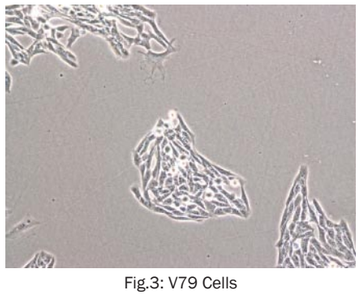


Fig.3: V79 Cells

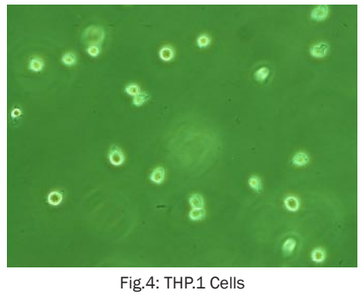


Fig.4: THP.1 Cells



Table 1: Test Items

| Cell | Evaluation Method | Test Principle; Test Item |
|---|---|---|
| PHK 16<br>(Epithelial Cell)<br>Epithelial Tissue | Cell Proliferation Test | Analysis method the index of which is the gradation from coloring viable cells only, using a reagent (WST-8)<br>→ Impact on cell enzyme metabolism |
| V79<br>(Fibroblasts)<br>Connective Tissue | Colony-Forming Assay | Analysis method the index of which is the number of colonies formed (cell colony)<br>→ Impact on cell colony formation |
| THP. 1<br>(Leukocyte)<br>Immune System | Trypan Blue Exclusion Test | Analysis method the index of which is the number of viable and dead cells stained by a staining reagent (Trypan Blue)<br>→ Impact on cell growth, life and death |
| — | Fuchsin Staining Test | Test method of staining monomers in the test material using staining reagent (Fuchsin Staining)<br>→ Relevance of the amount of monomer and the test result |

Each test is conducted as below.

(1) PHK16 Cell Proliferation Test (WST-8 Assay)

2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt(WST-8) was resolved by dehydrogenase (NAD+, NAD(P)+ dehydrogenase) that viable cells exhibit into the orange-colored WST-8 Formazan(Fig 5). It was possible to indirectly determine the number of viable cells by measuring the density of this orange color (Fig 6).

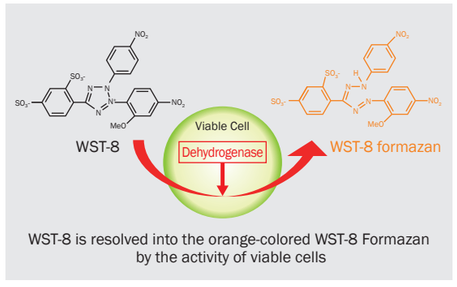



Fig. 5: WST-8 → WST-8 Formazan Assay Mechanism

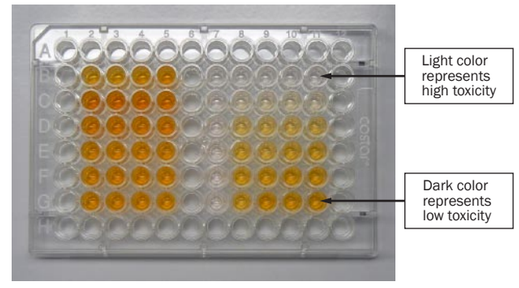
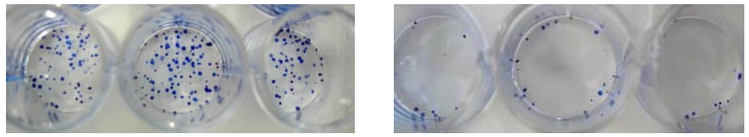



Fig. 6: Result of WST-8 Formazan Assay

A cell culture medium was added to a specimen so as to make the surface area/volume ratio 3cm²/ml and extracted for 72 hours in carbon gas incubation at 5% $CO_2$, 37°C. Sample solution (100% density) was made by sterilizing the extract with a 0.22$\mu$m filter. 10,000 PHK16 cells were distributed on each well of a 96-hole culture plate and incubated for 48 hours in carbon gas incubation. After exchanging culture medium, incubation proceeded for 72 hours, sample solution was added, and incubation was re-commenced for 48 hours. WST-8 sample solution was then added to each well, coloring took place for 4 hours, and absorptivity ratio was measured at 450mm. Taking the well as a reference value using the culture medium instead of sample solution, toxicity in the material was evaluated from the change in the absorptivity ratio against the reference value.

(2) V79-Cell Colony-Forming Assay

V79 cells form colonies as the cells grow. It is possible to evaluate toxicity from the number of colonies formed (Fig 7). The test was conducted in compliance with ISO 10993 “Biological Evaluation of Medical Devices” as shown below. A cell culture medium was added to a specimen so as to make the surface area/volume ratio 3cm²/ml and extracted for 72 hours in carbon gas incubation at 5% $CO_2$, 37°C. Sample solution (100% density) was made by sterilizing the extract with a 0.22$\mu$m filter.



No Toxicity　　Toxicity

Fig. 7: Comparison of Colonies

100 V79 cells were distributed on each well of a 24-hole culture plate. Incubation took place for 24 hours in carbon gas at 5% $CO_2$, 37°C and the culture medium was replaced with sample solution and incubated for 120 hours. Removing the culture medium from the wells, formaldehyde was added to harden cell colonies, and the cells were stained with Trypan Blue. The number of colonies formed was counted, with each colony containing more than 50 cells counted as 1.

(3) THP.1Cells Trypan Blue Exclusion Test

Trypan Blue (blue color) pigment compound is not absorbed by viable cells but is absorbed by dead cells, staining such cells blue (Fig 8). Taking advantage of this characteristic, the test is carried out in order to measure cell viability with regard to cell growth by observing viable and dead cells under a microscope (Fig 9).

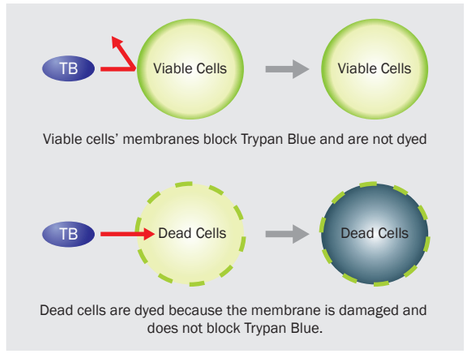



Fig. 8: Trypan Blue Dye Mechanism

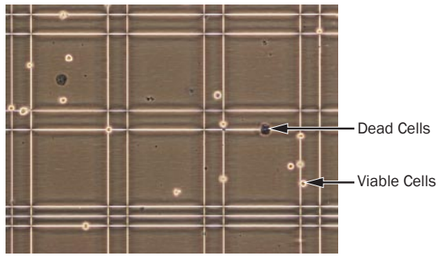



Fig. 9: Hemocytometer Measurement

Specimens were put in place and 100,000 THP.1 cells were distributed on each well of a 24-hole culture plate. Incubation took place for 72 hours in carbon gas at 5% $CO_2$, 37°C. After incubation, cell solution was collected, equal amount of Trypan Blue were added, and viable/dead cells were counted on a hemocytometer. The amount of cell increase during the 72 hour-incubation period (Cell Growth Rate) and the proportion of viable cells (Cell Viability) in the total number of cells (viable/dead cells) were evaluated.

(4) Fuchsin Staining Test

The fuchsin used in this test has the characteristic of staining with a red color by reacting with existing monomers in the specimens (Fig 10). The analysis method is to quantify the number of monomers in the material by measuring the color difference produced as a result of this staining.

Left: Light Staining Specimen, Right: Heavy Staining Specimen

Fig. 10: Fuchsin Staining

The specimens were immersed in a sufficient quantity of 0.2% W/V fuchsin solution and left for 24 hours at 37°C. After immersion, the specimens were collected, washed thoroughly with water, and the color (L*,a*,b*) before and after staining was measured using a colorimeter; the color difference was then evaluated and ascertained.



# 《 3. Test Results and Discussion 》

## 3.1 PHK16 Cell Proliferation Test

The result of the PHK16 Cell Proliferation Test shows that absorbance was plotted relatively evenly at the point when materials were added, and that the closer relative absorbance got to 100%, the lower the toxicity which was exhibited, taking absorbance of a reference value as 100%. As a result, no decrease of relative absorbance was confirmed at any density in the TWiNY extract (Fig 11-1).

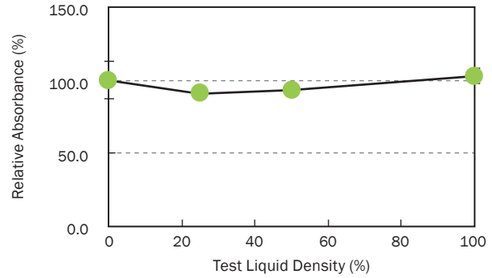



Fig. 11-1: PHK16 Cell Proliferation Test (TWiNY)

In the same way as for the TWiNY extract, no decrease of relative absorbance was confirmed in the Luna-Wing extract, which was used as a comparative subject. On the other hand, relative absorbencies in the test liquid at 50% and 100% density were 115% and 126% respectively, showing more than the reference value (Fig 11-2).

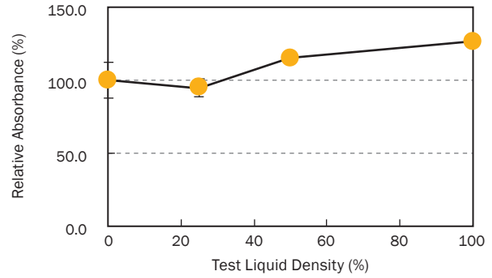



Fig. 11-2: PHK16 Cell Proliferation Test (Luna-Wing)

## 3.2 V79-Cell Colony-Forming Assay

The V79-Cell Colony-Forming Assay demonstrated that the closer the colony-formation ratio of a material gets to 100%, the lower toxicity it has. No decline in colony-formation ratio is confirmed at any density in the TWiNY extract (Fig. 12-1).

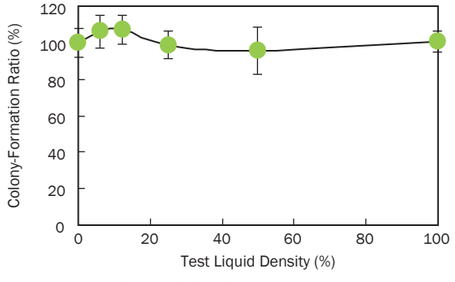



Fig. 12-1: V79-Cell Colony-Forming Assay (TWiNY)

In the same way as for the TWiNY extract, no decline in colony-formation ratio was confirmed at any density in the Luna-Wing extract, which was used as a comparative subject (Fig 12-2).

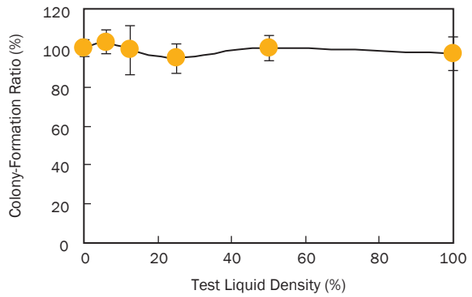



Fig. 12-2: V79-Cell Colony-Forming Assay (Luna-Wing)

## 3.3 THP.1 Cell Trypan Blue Exclusion Test

The result of the THP.1 Cell Trypan Blue-Exclusion Test is as displayed. The chart shows how many cells were propagated during the 3-day incubation period from the point that cells had been originally distributed (0 days) (Fig 13-1).

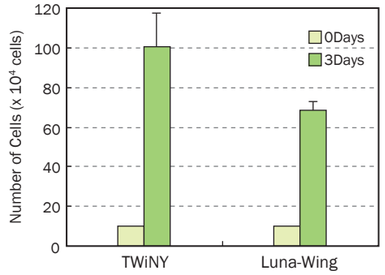



Fig. 13-1: THP.1 Cell Growth

The THP.1 cells incubated on TWiNY showed a much high propagation ratio, over 10 times that of the point cells originally distributed (0 days) (100,000 cells→1,070,000 cells). Moreover, the cell viability showed values of more than 90%. The THP.1 cells incubated on Luna-Wing, which is a comparative material, also showed a high propagation ratio, a little less than 7 times (100,000 cells→690,000 cells). More than 90% of cells were counted as viable; this showed the same high cell viability as for TWiNY. In the test using PHK16 cells (epithelial cells) and V79 cells (fibroblasts), no difference in impact on cells was confirmed between the materials, TWiNY and Luna-Wing. On the other hand, in the test using THP.1 (leukocyte), the cell growth incubated on TWiNY excelled that incubated on Luna-Wing, the number of cells being approximately 1.4 times that incubated on Luna-Wing. Luna-Wing has shown favorable results through various *in vitro* and *in vivo* examinations from component materials to basic resin, and is considered to be a high-safety dental prosthesis material product. The result of the THP.1 cell incubation suggests that TWiNY exhibits an even higher level of safety as a dental material than Luna-Wing. The causes why the cell growth of THP.1 cells incubated on TWiNY excelled that of those incubated on Luna-Wing were examined. 1) Component materials and 2) the manufacturing method are considered to be the causes of the differences between the materials (light and heat-cured indirect composite resin and indirect composite resin). For 1) Component materials, no particular difference between the materials with regard to influence on THP1 cells materials were confirmed. Therefore, 2) the manufacturing method was examined further. As shown in the material, the major difference of manufacturing method between the light and heat-cured indirect composite resin, TWiNY and the Luna-Wing, indirect composite resin is whether heat cure is performed or not after light cure. At each curing procedure, uncured monomers remain when the monomers in the component materials are being cured to polymers. Research on dental polymer materials reports that monomers which are component materials have an impact on cells. Monomers such as urethane dimethacrylate (UDMA) and triethylene glycol dimethacrylate (TEGDMA), have achieved long-term clinical use as the main components of indirect composite resin. These monomers are used in many products on the market, including our products, TWiNY and Luna-Wing.



It is assumed that TWiNY does not impact THP.1 cells because TWiNY, on which heat cure is performed after light cure, has a smaller quantity of uncured monomers compared to Luna-Wing, on which only light cure is performed.

Besides TWiNY and Luna-Wing, TWiNY (–), for which heat curing is omitted and Luna-Wing (+), for which heat curing is carried out, were prepared and were used on the THP.1 cells in a further Trypan Blue Exclusion Test. On analyzing the THP.1 cells incubated on TWiNY and TWiNY(–), the number of cells incubated on TWiNY(–) was found to have greatly decreased as a result of omitting heat curing (51% compared to TWiNY). Cell viabilities for both TWiNY and TWiNY (–) were over 90% and almost the same as the reference value (Fig 13-2).

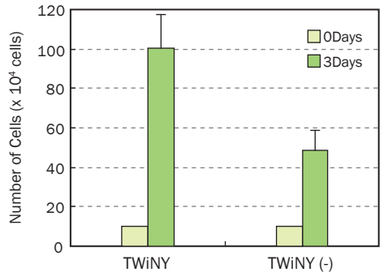



Fig. 13-2: THP. 1 Cell Growth

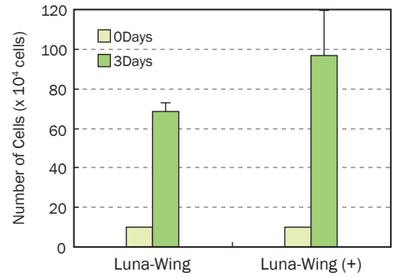



Fig. 13-3: THP. 1 Cell Growth

Next, the same test was conducted on Luna-Wing and Luna-Wing (+). After an additional heat curing had been performed, incubation was carried out on Luna-Wing (+); the number of cells was found to have greatly increased, exhibiting a sustained high cell viability (over 90%) (a 40% increase over Luna-Wing, Fig13-3). These results support the hypothesis that uncured monomers influence THP.1 cells.

## 3.4 Fuchsin Staining Test

In order to examine the influence of uncured monomers on THP.1 cells in more detail, uncured monomers found in the four materials tested in the Trypan Blue Exclusion Test (TWiNY, TWiNY (–), Luna-Wing and Luna-Wing (+)) were stained with fuchsin. In the test, the more uncured monomers there were in the materials, the larger degree of staining (pigment) by fuchsin was recorded. The relationship between the two measured results was analyzed by showing the results of cell examinations and staining tests graphically for each material.

As a result, a high correlativity, $R^2$=0.6659, was confirmed between the color differences and the cells (Fig 14). Since the color differences correspond to the quantity of uncured monomers, this result shows a strong involvement between uncured monomers and THP.1 cells.

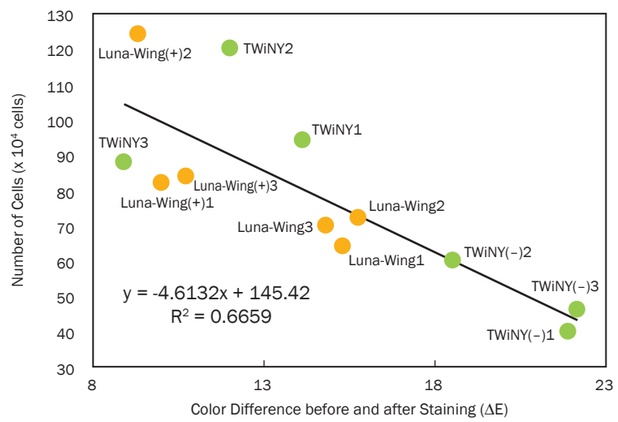



Fig. 14: Correlation Diagram of the Number of Cells and Color Differences

## 《 4. Conclusion 》

The cell tests were conducted using the light and heat-cured indirect composite resin TWiNY and the indirect composite resin Luna-Wing, for three kinds of cells assuming conditions in the mouth: PHK16 cells, V79 cells, and THP.1 cells. No toxicity was confirmed in TWiNY and Luna-Wing for PHK16 cells (epithelial cells) and V79 cells (fibroblasts).

No induction of cell death was confirmed in TWiNY and Luna-Wing for THP.1 cells, which are immune cells. Moreover, cell growth on TWiNY was found to be superior to that on Luna-Wing. It is considered that this difference occurs because by performing heat cure, TWiNY has fewer uncured monomers that display cell proliferation activation than Luna-Wing has.

Reports and articles on the safety of Luna-Wing have hitherto been published on a number of occasions. No trouble related to human physical safety has been reported with regard to Luna-Wing since it was released onto the market. TWiNY is considered to be a higher-safety product than Luna-Wing, which has also displays a high level of safety, as mentioned.

Testing was undertaken in collaboration with the Dental Surgery Science Course, Kochi Medical School, Kochi University.

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