# HALOLINK™ RESIN FOR PROTEIN PULL-DOWN AND ANALYSIS

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HaloLink™ Resin provides a method for specific, covalent and oriented immobilization of proteins onto surfaces. Here we demonstrate the high binding capacity and minimal nonspecific binding of the HaloLink™ Resin. We also show that the covalent HaloTag™ bond provides stability during dilution and stringent washing, minimizing the loss of the HaloTag™ fusion proteins from the surface. By analyzing protein:protein interactions and enzyme activity for several fusion proteins, we show that HaloTag™ fusion proteins bound to the HaloLink™ Resin maintain function. HaloLink™ Resin is not a purification resin; however, in conjunction with protease cleavage, it can be used to isolate a protein of interest.

## Introduction

Proteomics is a rapidly growing field characterized by developing technologies for studying proteins and their functions. The challenge that we face is to elucidate the function of all proteins and how they assemble into complex networks responsible for key cellular processes. Surface-based proteomics tools will play an important role in this effort. Surface-based proteomics requires general and facile methods for immobilizing proteins on solid surfaces in known orientations without disrupting protein structure or function. Furthermore, this immobilization must exhibit high binding capacity and minimal nonspecific adsorption (1).

**The new HaloLink™ Resin enables covalent and oriented attachment of HaloTag™ fusion proteins to a solid surface.**

The HaloLink™ Resin[(a)] enables covalent and oriented attachment of HaloTag™ fusion proteins to a solid surface. The resin comprises HaloTag™ Ligand linked to a Sepharose® surface. The binding capacity is high, and nonspecific binding is low. The covalent nature of the HaloTag™ bond provides stability during dilution and stringent washing, minimizing the loss of the HaloTag™ fusion proteins from the surface. We demonstrate the utility of the HaloLink™ Resin in several different applications including protein:protein interactions in vivo and in vitro, analysis of enzymatic activity of proteins on the surface and purification of proteins of interest in conjunction with specific protease cleavage. Immobilizing proteins onto a solid support using the HaloLink™ Resin is a simple procedure that can be completed in 60 to 90 minutes (Figure 1). This method is optimized for proteins expressed in cell-free expression systems (in vitro) or in mammalian cells.

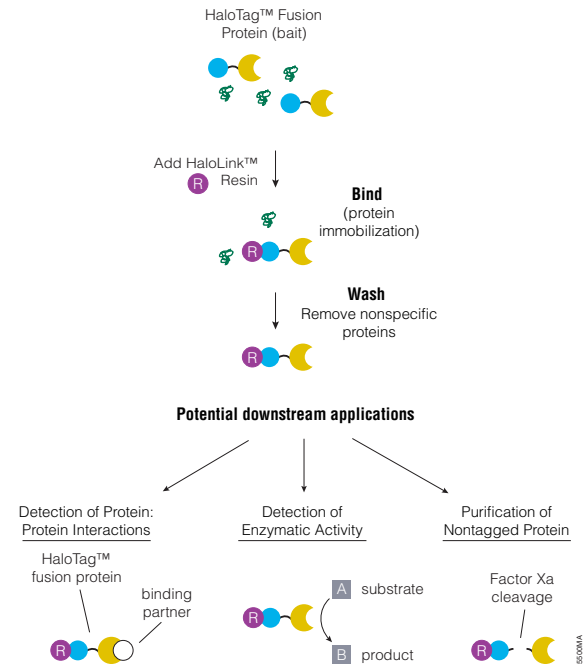


**Figure 1. Overview of the HaloLink™ Resin immobilization protocol and potential downstream applications.**

## HaloLink™ Resin: High Binding Capacity, Specificity and Stability of Protein Binding

An important feature of any affinity binding resin is its binding capacity. Since HaloLink™ Resin is an immobilization resin and not a purification resin, we estimate binding capacity by the amount of the protein bound to the resin rather than by the amount of eluted protein. Based on such experiments, we have determined the binding capacity to be at least 7mg of HaloTag™ fusion protein per milliliter of settled resin. This is comparable to the binding capacities of other affinity binding resins.

Another important feature of affinity resins is minimal nonspecific protein binding. This aspect is especially important when the resin is used for analysis of protein:protein interactions in pull-down assays. We

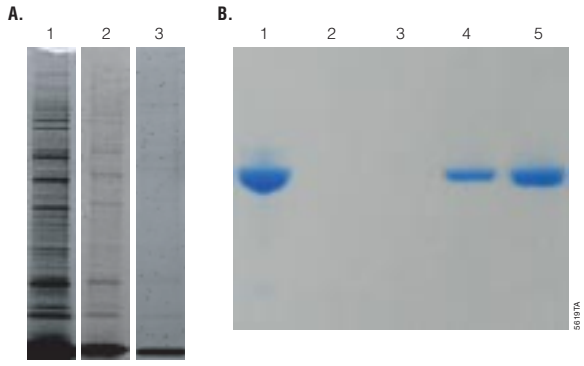


**Figure 2. Specific and stable binding of HaloTag™ fusion proteins to HaloLink™ Resin. Panel A.** Low nonspecific binding of proteins to HaloLink™ Resin. HaloLink™ Resin (50µl) was treated with 400µg rabbit reticulocyte lysate according to the binding protocol in reference 5, boiled in SDS loading buffer, and the supernatant was analyzed by SDS-PAGE (lane 3). Sypro®Ruby Fluorescent Dye (Molecular Probes Cat.# 12000) was used to detect proteins. Lane 1, 500-fold diluted rabbit reticulocyte lysate; lane 2, 1,000-fold diluted rabbit reticulocyte lysate. **Panel B.** HaloTag™-GST fusion protein stably binds to the HaloLink™ Resin. Equivalent amounts of settled HaloLink™ Resin and GST binding resin (25µl) were incubated with 160µg of HaloTag™-GST fusion protein; resins were washed and resuspended in PBS. After incubation at 4°C, supernatants were removed and analyzed by SDS-PAGE. Lane 1, 10% of the input HaloTag™-GST fusion protein; lane 2, supernatant from the HaloLink™ Resin removed at 10 minutes; lane 3, supernatant from the HaloLink™ Resin removed at 24 hours; lane 4, supernatant from GST-binding resin removed at 10 minutes; lane 5, supernatant from the GST-binding resin removed at 24 hours.

evaluated nonspecific binding by treating HaloTag™ Resin with rabbit reticulocyte lysates used for in vitro protein expression. We boiled the resin in SDS loading buffer to remove nonspecifically bound proteins and analyzed the supernatant along with dilutions of lysate used in the reaction. The lane containing the supernatant of resin boiled in SDS buffer has almost undetectable amounts of protein, indicating that nonspecific binding of HaloLink™ Resin is low (Figure 2, Panel A).

Many applications used for protein analysis require extensive washing to remove nonspecifically bound proteins. A unique feature of HaloLink™ Resin is that proteins are bound to the resin covalently, which allows users to wash away nonspecifically bound proteins while retaining the fusion protein. To demonstrate this point, we bound HaloTag™-GST fusion protein to either HaloLink™ Resin or to a GST-binding resin. Following binding and washing, the resins carrying bound proteins were resuspended in phosphate-buffered saline (PBS). After an incubation period, we removed supernatant and analyzed it for the presence of the protein. The resin was resuspended in buffer once more, and after an additional 24-hour incubation, the second supernatant was analyzed. Results of the experiment demonstrate that HaloTag™-GST protein is stably attached to the HaloLink™ Resin, since protein was not detected in the supernatant; in contrast the HaloTag™-GST fusion protein leaches from the GST-binding resin with each dilution (Figure 2, Panel B).

## Detecting Protein:Protein Interactions

Pull-down assays probe interactions between a fusion protein consisting of an affinity tag and protein of interest (bait) and potential interacting partners (prey) in solution. Bait-prey complexes are isolated from solution by specific binding of the bait to the affinity resin. The most common pull-down technique is based on the GST affinity tag (2).

We performed pull-down experiments using HaloTag™ Protein and HaloLink™ Resin. The properties of the HaloTag™ Protein provide some important advantages that increase chances of successful isolation of interacting partners. First, the HaloTag™ Protein provides covalent attachment to the resin, allowing extensive washing to remove nonspecifically bound proteins. Second, HaloTag™ Protein binds the resin rapidly with high affinity. These features allow efficient immobilization of proteins present at low concentrations (e.g., proteins expressed in cell-free expression systems). Thus HaloTag™ Protein, in conjunction with HaloLink™ Resin, uniquely enables detection of protein interactions when both proteins are expressed in vitro.

We used the well characterized interacting partners c-Jun and c-Fos (3) to demonstrate pull-down using the HaloLink™ Resin. The c-Fos-HaloTag™ fusion was created by inserting the c-*fos* sequence into pFC8A vector (4); the c-*jun* sequence was inserted into pF1A Vector (Cat.# C8441, 4). Both proteins were expressed separately in vitro. The c-Jun (prey) was labeled with [$^{35}$S]-methionine to aid detection. The HaloLink™ Resin was added to the cell-free reaction expressing the c-Fos-HaloTag™ fusion protein (bait), allowing immobilization of the bait on the resin. We set up a control reaction containing the HaloLink™ Resin and cell-free expression system without the bait. The cell-free expression reaction [$^{35}$S]-labeled c-Jun was added to both resins: the experimental sample (containing the bait) and the control (no bait). The bait and the prey were allowed to interact and then washed with buffer containing a low amount of nonionic detergent (5). To elute the captured prey, the resin was resuspended in SDS loading buffer, boiled and analyzed. The results in Figure 3 show that the prey protein was recovered only from the resin carrying the bait and not from the control. Thus the pull-down of c-Jun by the c-Fos-HaloTag™ fusion protein was efficient and specific as judged by absence of any prey protein in the control lane.

## Detecting Regulated Protein:Protein Interactions

In the pull-down experiments described above, we precharged the HaloLink™ Resin with the bait protein before adding the prey. Next, we asked two additional questions. First, could we efficiently detect protein:protein interactions when the protein binding partners are allowed to interact first and then the

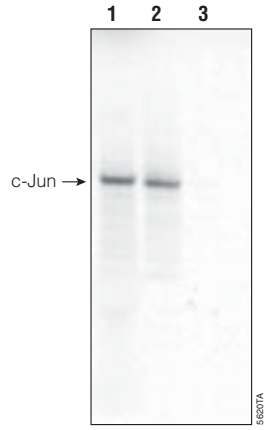


**Figure 3. Detecting protein interactions in a pull-down experiment.** The c-Fos-HaloTag™ fusion protein was expressed in the TNT® T7 Quick Coupled Transcription/Translation System (Cat.# L1170); 20µl of the TNT® reaction containing the c-Fos-HaloTag™ fusion was added to 50µl of the HaloLink™ Resin; immobilization was carried out according to the provided protocol (5). Prey protein (c-Jun) was synthesized and labeled with [$^{35}$S]-methionine in vitro, and 20µl of the TNT® reaction was added to the HaloLink™ Resin carrying the c-Fos-HaloTag™ fusion. A parallel control was performed in which prey was added to the resin without the bait. After incubation and extensive washing [five 1ml wash steps using 0.05% IGEPAL® (Sigma Cat.# CA-630) with 1% BSA], proteins were eluted by boiling in SDS loading buffer and separated by SDS-PAGE. Results were scanned on a Typhoon® Imager. Lane 1, in vitro reaction containing expressed $^{35}$S-methionine-labeled c-Jun; lane 2, pull-down reaction c-Fos-HaloTag™:cJun; lane 3, control reaction.

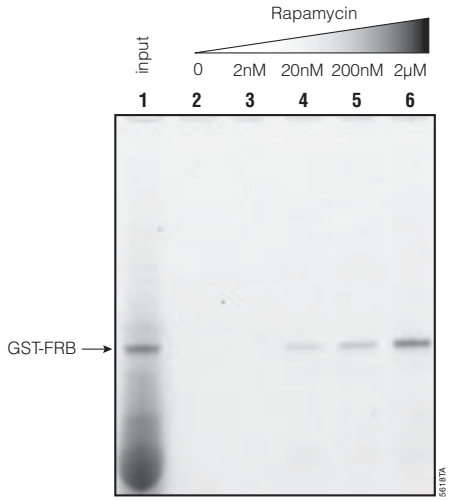


**Figure 4. Detecting regulated protein:protein interactions.** GST-FRB and HaloTag™ FKBP fusion proteins were expressed using the TNT® T7 Quick Coupled Transcription/Translation System (Cat.# L1170). FluoroTect™ Green$_{Lys}$ Labeling System (Cat.# L5001) was used to label the prey. The two reactions were mixed (20µl) in the presence of the indicated amounts of rapamycin. Samples were incubated 1 hour to allow proteins to bind, and then HaloLink™ Resin (50µl) was added to pull down the complexes. The reactions were incubated for 1 hour at room temperature with mixing; prey protein was eluted with SDS loading buffer and analyzed by SDS-PAGE. A Typhoon® scanner was used to detect fluorescent signal. Lane 1, input in vitro TNT® reaction containing GST-FRB; lane 2, control reaction in the absence of rapamycin; lanes 3–6, reactions containing rapamycin.

complexes are pulled down by adding the resin? Second, could we follow formation of protein:protein interactions that are modulated by a small molecule?

To test these parameters we analyzed the interaction between FK506 binding protein (FKBP) and the FKBP-rapamycin binding domain of mTOR (FRB) proteins (6–8). Rapamycin is needed for interaction between FKBP and FRB; FRB binds to a preformed FKBP-rapamycin complex (6). Formation of the ternary complex (FKBP-rapamycin-FRB) can be modulated by changing rapamycin concentration. An increase in rapamycin concentration results in increased FKBP-FRB interaction, predicting increasing amounts of the prey protein (FRB) in the pull-down reaction as rapamycin concentration increases. Also, FRB should not be detected in the absence of rapamycin.

FKBP protein was inserted into the pFC8A Vector to create a C-terminal HaloTag™-FKBP fusion protein. The FRB protein was expressed as a GST fusion from pFN2A (Cat.# C8461; 4) plasmid. After incubation, protein complexes were subsequently pulled down by HaloLink™ Resin. Both proteins were expressed in a cell-free system and mixed in the presence of various concentrations of rapamycin. Figure 4 shows that recovery of the fluorescently labeled prey protein depends on the concentration of rapamycin in the binding reaction and that no FRB was detected in the absence of rapamycin.

Successful detection of protein:protein interactions largely depends on the concentration of bait protein bound to the solid support. Therefore in most cases, bait proteins are synthesized in bacteria to assure high concentration. Proteins synthesized in the cell-free expression systems are usually present at much lower amounts. However bacterial expression is time consuming, and many mammalian proteins are difficult to express in bacteria. Thus, using cell-free protein synthesis is faster and increases chances of obtaining soluble protein. As shown above, HaloLink™ Resin enables efficient detection of protein interactions when both proteins, the bait and prey, are synthesized in vitro. We compared the efficiency of GST pull-down compared to HaloLink™ Resin pull-down using in vitro-synthesized proteins. The protein pair c-Jun:c-Fos was used in these experiments; c-Fos was fused to GST and c-Jun was fused to HaloTag™ Protein. Thus the same protein pair can be isolated either by using GST binding resin or HaloLink™ Resin (Figure 5). HaloLink™ Resin proved to be the more efficient and specific method. The amount of prey protein isolated by HaloLink™ Resin is higher than that isolated using GST resin. Additionally, the control sample shows considerable amount of nonspecific binding of prey to GST resin, while in the case of HaloLink™ Resin the nonspecific binding is not detectable.

# HaloLink™ Resin for Protein Pull-Down and Analysis

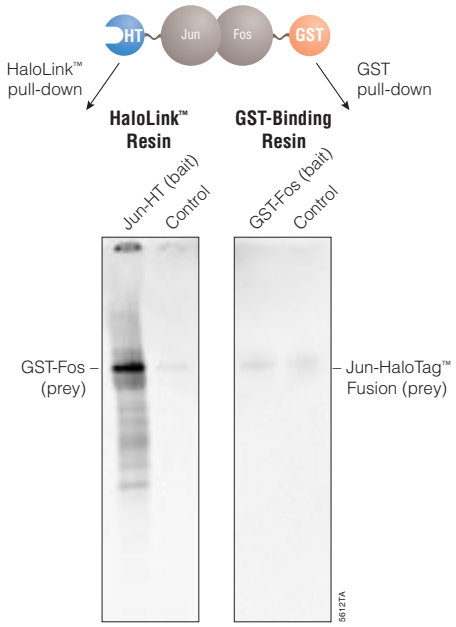


**Figure 5. Comparison of HaloLink™ Resin and GST pull-down methods.** GST-c-Fos and c-Jun-HaloTag™ fusion proteins were synthesized in a cell-free system and either left unlabeled to serve as bait, or labeled with $^{35}$[S]-methionine to serve as prey. After synthesis was complete, the reactions were mixed in two sets, each consisting of an unlabeled protein and a labeled protein partner, to allow the proteins to bind. The appropriate protein complexes were pulled down by either HaloLink™ Resin (**left**) or by GST-binding resin (**right**). Bound: GST-c-Fos or c-Jun-HaloTag™ protein pull-down on HaloLink™ or GST resin, respectively; Control: HaloLink™ or GST resin only.

## Detecting Protein Interactions in vivo

While detecting protein interactions in vitro is important for understanding protein function, it is equally important to be able to analyze protein interactions in vivo. We used the interacting proteins p65 and IκB to demonstrate that HaloLink™ Resin can be used to isolate protein complexes from mammalian cells. HeLa cells were transiently transfected with pFC8A Vector encoding p65-HaloTag™ fusion protein. Cells were collected and lysed using mechanical disruption 24 hours post-transfection. Resin was added to the lysed transfected cells and also to control reactions containing non-transfected cells. Proteins covalently attached to HaloLink™ Resin were analyzed by Western blot using antibodies directed against IκB. Results in Figure 6 demonstrate that IκB could be detected only in transfected cells as a result of IκB-p65-HaloTag™ fusion interactions and not in nontransfected cells that do not contain the p65-HaloTag™ fusion partner.

## Immobilizing Enzymes onto HaloLink™ Resin

HaloLink™ Resin can also be used to capture and covalently immobilize enzymes from complex protein mixtures. We tested whether enzymes maintain their activity once attached to the surface by measuring enzymatic activity of luciferase-HaloTag™ fusions. The luciferase-HaloTag™ fusion and

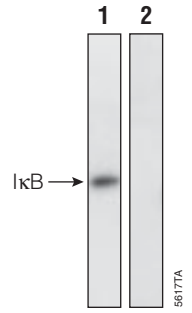


**Figure 6. Detecting protein:protein interactions in vivo.** HeLa cells were transfected with pFC8A carrying p65-HaloTag™ fusion protein. Cells were collected in 1ml of buffer 24 hours post-transfection and lysed using a glass homogenizer. HaloLink™ Resin (100µl) was added to 100µl of the total cell lysate. Nontransfected cells were also processed to serve as a negative control. Resin was incubated with the cell lysate for 1 hour at room temperature, washed, and prey protein was eluted with SDS loading buffer. Proteins were resolved by SDS-PAGE and analyzed by Western blot using antibodies directed against IκB. Lane 1, protein prepared from transfected cells eluted from resin; lane 2, control sample (nontransfected cells).

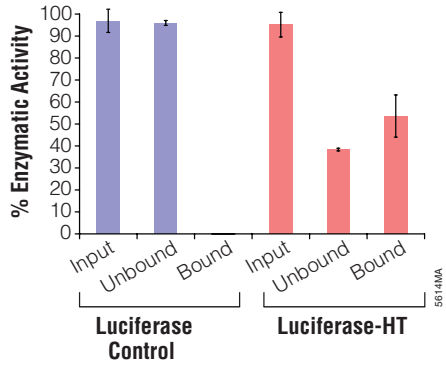


**Figure 7. Detecting enzymatic activity of proteins immobilized onto the HaloLink™ Resin.** Luciferase-HaloTag™ fusion protein was synthesized in vitro, and 20µl of the reaction was added to 50µl of the HaloLink™ Resin. To assess the specificity of attachment, nontagged luciferase was expressed and added to the resin as a negative control. The graph shows the percent of enzymatic activity in different fractions. The input material (in vitro transcription/translation reaction) has 100% enzymatic activity; the enzymatic activity in the unbound fraction and the activity on the resin are 40% and 60%, respectively.

luciferase without the tag were expressed in vitro. HaloLink™ Resin was added to both extracts, and proteins were immobilized according to the protocol described in the Technical Manual (7). Over 60% of the total enzymatic activity of the luciferase-HaloTag™ fusion was detected on the HaloLink™ Resin (Figure 7), indicating that the bound fusion protein retained enzymatic activity. The rest of the activity was detected in the unbound fraction. These results demonstrate that proteins immobilized onto the HaloLink™ Resin maintain their native conformation and enzymatic activity. As expected, no activity is associated with resin that was incubated with luciferase not fused to the HaloTag™ Protein; thus binding to resin is specific and HaloTag™ Protein-dependent.

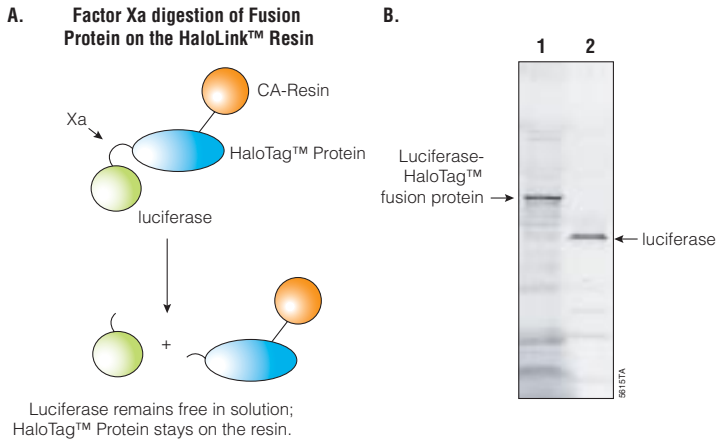


**Figure 8. Release of fusion protein off the HaloLink™ resin by proteolytic cleavage. Panel A.** Schematic showing use of Factor Xa cleavage to release fusion protein from the HaloLink™ Resin. **Panel B.** Luciferase-HaloTag™ fusion was expressed in vitro and labeled with the FluoroTect™ $Green_{Lys}$ Labeling System (Cat.# L5001). A 20µl aliquot of the reaction was added to 50µl of HaloLink™ Resin and incubated for 30 minutes at room temperature followed by washing (7). Resin carrying luciferase-HaloTag™ fusion on the surface was treated with Factor Xa for 1 hour at room temperature; supernatant was collected and analyzed for the presence of nontagged luciferase by SDS-PAGE and scanned on a Typhoon® imager. Lane 1, 4µl of in vitro transcription/translation reaction containing luciferase-HaloTag™ fusion protein; lane 2, supernatant collected after Factor Xa cleavage; 4µl of the 50µl cleavage reaction was loaded.

## Purifying Proteins Using HaloLink™ Resin

HaloLink™ Resin allows permanent attachment of proteins to the resin. Such permanent attachment does not allow purification of the HaloTag™-fusion proteins by simple elution. However, the pFC8A and pFC8K Vectors contain a protease cleavage site (Factor Xa) situated in the linker sequence between the HaloTag™ Protein and the protein of interest. This allows release of the protein of interest from the HaloLink™ Resin by Factor Xa protease cleavage. Because the cleavage is performed on the protein bound to the resin, any contaminating proteins can be removed by extensive washing before the cleavage reaction, and the released target protein is tag-free. We demonstrated this using a luciferase-HaloTag™ fusion protein expressed in vitro. Following immobilization, the fusion protein was cleaved with Factor Xa. The supernatant was collected and analyzed. Release of luciferase without the tag is demonstrated in Figure 8.

## Summary

HaloLink™ Resin provides a convenient way to covalently immobilize HaloTag™ fusion proteins on a solid support in an oriented fashion. We show that HaloLink™ Resin enables studies of protein function in a variety of assays. Immobilized proteins can be used in protein pull-down assays to isolate protein binding partners in vivo (mammalian cells) or in vitro, or they can be evaluated for their enzymatic activity. Target proteins can be released from the resin by proteolytic cleavage, resulting in pure protein. Immobilization of proteins onto HaloLink™ Resin is covalent, rapid and selective, allowing efficient binding of proteins without purification or protein modification prior to immobilization. These characteristics make the HaloTag™ Protein and HaloLink™ Resin well suited for studies of protein functions in vivo and in vitro. ■

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## Protocol

*HaloLink™ Resin Technical Manual* #TM250, Promega Corporation.
(www.promega.com/tbs/tm250/tm250.html)

## Ordering Information

| Product | Size | Cat.# |
|---|---|---|
| HaloLink™ Resin | 2ml | G1911 |
| | 5ml | G1912 |

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