The SureVector Cloning System

Technical Note

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Introduction

Molecular cloning is a ubiquitous technique essential to all fields of biological and medical research. It is frequently a foundational step in a wide range of workflows, including understanding gene function and regulation, protein expression, and functional characterization of macromolecules. However, molecular cloning can often be rate-limiting due to the time and effort required to assemble functional DNA constructs. Additionally, the breadth of applications leads to extensive variation in requirements for vector composition in individual experiments.

Agilent's SureVector system enables the rapid and reliable assembly of multiple DNA modules into a recombinant plasmid containing your target gene. These DNA modules consist of functional components (selection markers, promoters, etc.) that make up existing cloning vectors. In a modular synthetic biology approach, these synthons can then be combined at will in a validated, ordered manner. SureVector harnesses the power of synthetic biology's standard parts to assemble custom vector constructs, and is designed with an easy-to-use web interface where you can configure any of the thousands of buildable vectors. Once you have the kit in your lab, a custom plasmid containing your gene-of-interest is just a 20 minute reaction away.

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Key advantages of the innovative SureVector system include:

- Rapid custom vector generation – less than a day from design to vector, compared to 4 weeks for custom vector services
- Enhanced flexibility – assemble new vectors quickly as experimental requirements change, rather than having to order new ones
- Control of experiments – get the construct you want when you need it, rather than settling for what a catalog can provide
- Reliable and precise assembly – SureVector has been extensively validated to ensure that standard parts can be interchanged without loss of functionality, and it is the only next-generation plasmid assembly technology to guarantee assembly of multiple functional DNA fragments

This technical overview illustrates the utility and reliability of the SureVector system by demonstrating the validation of the system for construction of bacterial, mammalian and yeast cloning vectors.

The Kit

The SureVector Core Kit has seven components:

- 16 DNA modules divided into 6 functional groups

Promoter-Tag	XP1 Expansion	Selectable Marker
XP2 Expansion	Origin-of-Replication	Gene-of-Interest (GOI)

- *Dpn* I enzyme
- SureVector Enzyme mix
- SureVector buffer (10x)
- dNTPs
- SureSolution (5x)
- XL1-Blue Chemically Supercompetent Cells

The SureVector core kit provides functionality in *E. coli*, yeast, and mammalian cells (Figure 1). The two expansion slots (XP1 and XP2) expand utility beyond bacteria to yeast and mammalian systems. If the yeast and mammalian modules are not used, the XP1 and XP2 linkers are used to close the plasmid circle. Also included in the core kit (p/n G7514A) are all of the buffers, enzymes and nucleotides required to generate SureVector plasmids, as well as XL1-Blue supercompetent cells. Assembly of a SureVector plasmid from Agilent's standardized DNA modules takes only 20 minutes in a single-tube reaction. Total hands-on time including transformation is less than two hours. With the SureVector core kit, up to 216 different vectors can be created, and all steps downstream from the assembly are the same as traditional cloning procedures. The ease with which custom SureVectors can be built integrates seamlessly with your existing workflow. While SureVector offers "scarless", one-pot, rapid vector assembly like alternative next-gen systems, it is much more efficient when assembling more than 5 modules, and in fact as many as 7 modules can be assembled effectively using SureVector.

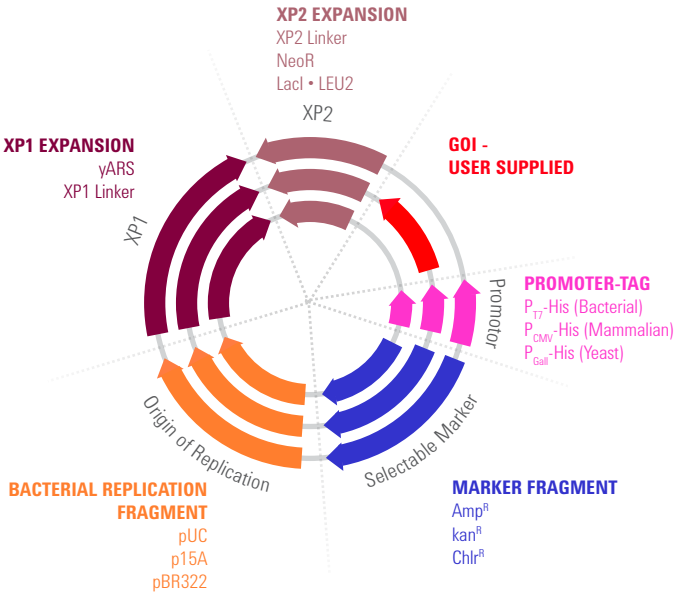

Figure 1. Structure of the SureVector cloning system.

Efficient and High Fidelity Assembly

Four test vector assemblies were generated which utilized all of the modules in all six DNA functional groups at least once (Table 1). The vectors were assembled *in silico* using the SureVector software tool (http://www.genomics.agilent.com/surevector) prior to the actual assembly process (Figure 2). For each of these assemblies, lacZ was inserted into the GOI slot as a positive control. The negative control was assembled in the absence of a GOI. In this case, a circular plasmid should not be formed, and very few transformed colonies should be observed.

Figure 3 shows the results of the transformations performed with the four test assemblies. In each case and across all three operators performing the experiment, transformation efficiency was very high using the lacZ gene as the GOI, and the number of colonies was verylow or non-existent in the absence of the GOI (negative control).

Three colonies for each assembly and each scientist were chosen at random and sent for sequence verification. Table 2 gives a summary of the sequencing results. Of the 216 junctions sequenced, three showed mutations and four failed to assemble correctly. Overall, 97% of the junctions had the correct sequence, and the percentage of correct junctions varied from 92 to 100%, across the three users.

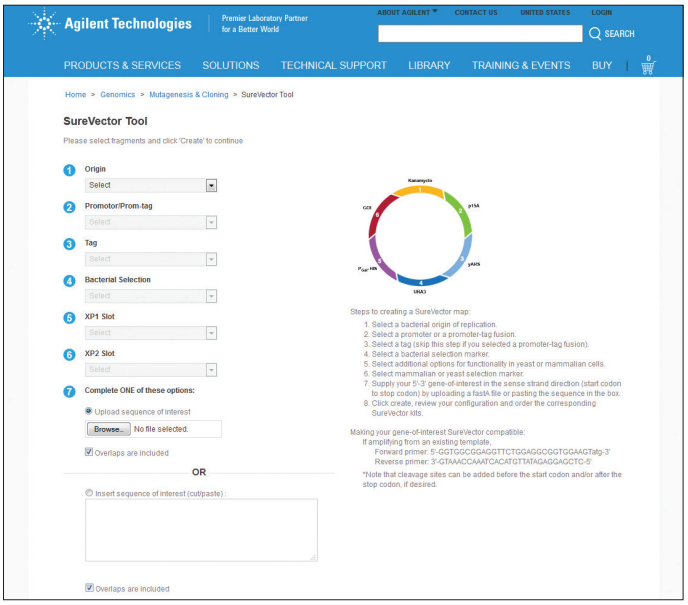

Figure 2. User interface for the SureVector design tool.

Assembly		Bacterial Selection Maker	*E. coli* Origin of Replication	Expansion Position #1	Expansion Position #2	Promoter/Tag
1	**Negative Control**	Amp^R	pUC	XPLinker1	LacI	P_{T7}-HIS_6
	With GOI†	Amp^R	pUC	XPLinker1	LacI	P_{T7}-HIS_6
2	**Negative Control**	Amp^R	pBR322	XPLinker1	NeoR	P_{CMV}-HIS_6
	With GOI	Amp^R	pBR322	XPLinker1	NeoR	P_{CMV}-HIS_6
3	**Negative Control**	Cam^R	p15A	yARS	LEU2	P_{GAL1}-HIS_6
	With GOI	Cam^R	p15A	yARS	LEU2	P_{GAL1}-HIS_6
4	**Negative Control**	Kan^R	p15A	XPLinker1	XPLinker2	P_{T7}-HIS_6
	With GOI	Kan^R	p15A	XPLinker1	XPLinker2	P_{T7}-HIS_6

†Gene-of-Interest (GOI)

Table 1. Four test vector assemblies that utilize all DNA functional groups at least once, with lacZ as the gene-of-interest.

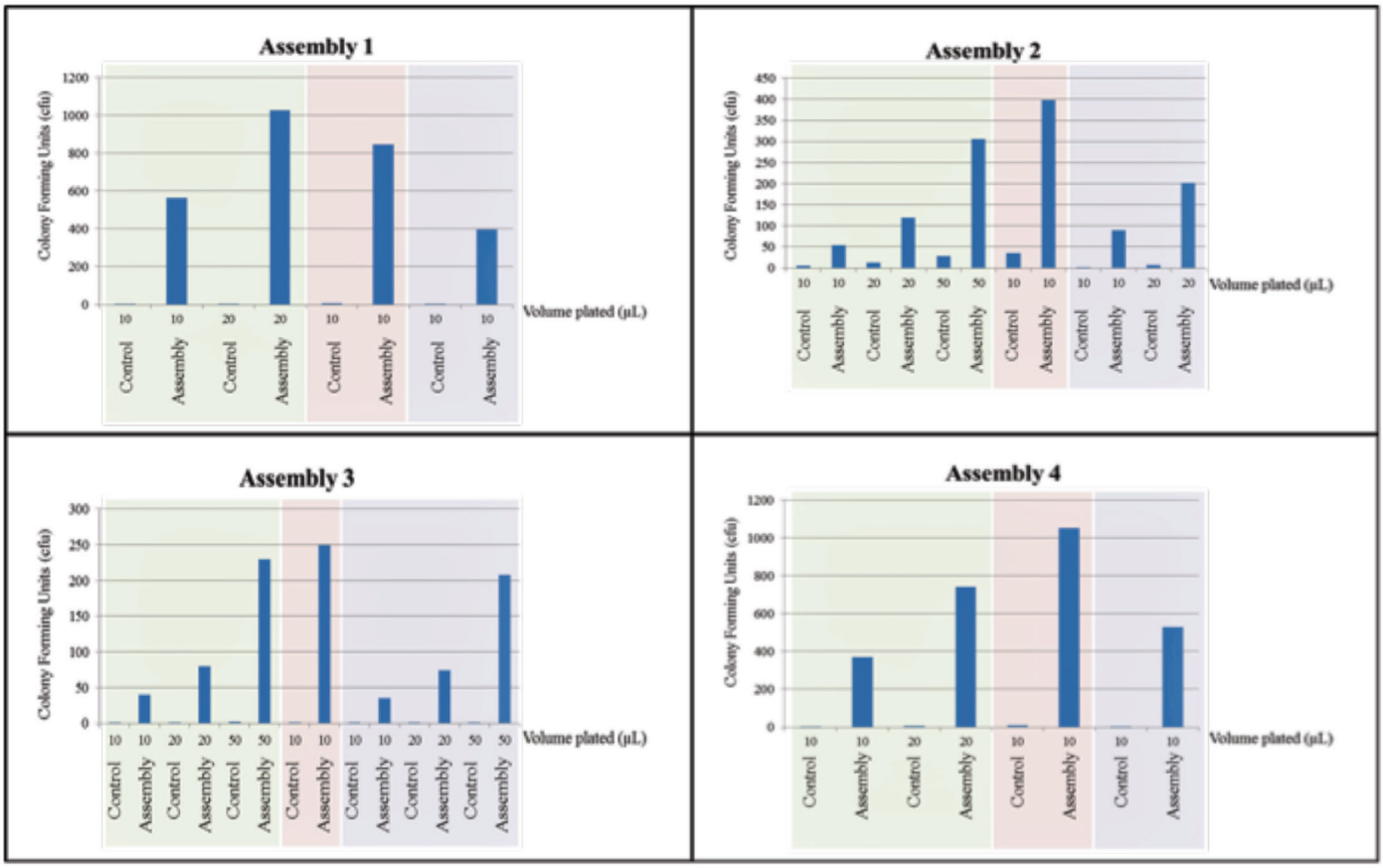

Figure 3. Transformation results (blue colonies) from four vector assemblies that collectively used every DNA module at least once (Table 1). The different color shades denote the three different operators that performed the assemblies and transformations.

	Operator #1			Operator #2			Operator #3			Totals
No. of Correct Junctions	24	24	23	24	24	24	24	23	19	**209**
No. of Incorrect Junctions	0	0	1	0	0	0	0	1	7	**7**
% of Correct Junctions	99%			100%			92%			**97%**

Table 2. Summary of sequence data from the junctions of the four test vector assemblies.

Functionality in Bacterial Systems

Three vector assemblies were used to demonstrate functionality in bacterial systems using the P_{T7}-HIS_6 promoter module, which contains the widely used T7 promoter as well as a histidine tag for subsequent protein purification, and the lac operator for inducible expression of the GOI (Table 3). The negative control did not contain a GOI, and the positive control utilized lacZ. The third vector construct contained the Septin2 gene, which codes for a 50 kDa mammalian protein. All three assemblies were transformed into Agilent BL21-Gold (DE3) competent cells and spread onto either LB + kanamycin (uninduced) or LB + kanamycin + IPTG (induced) plates.

Figure 4 shows SDS-PAGE gels of total cell protein samples taken from cultures of bacteria transformed with the Septin2 vector. Samples were collected at different time points before and after induction with two different concentrations of IPTG. In the presence of IPTG an induced protein of the correct size (about 50 kDa) can be seen which is absent in the uninduced sample (-), confirming inducible expression of the GOI.

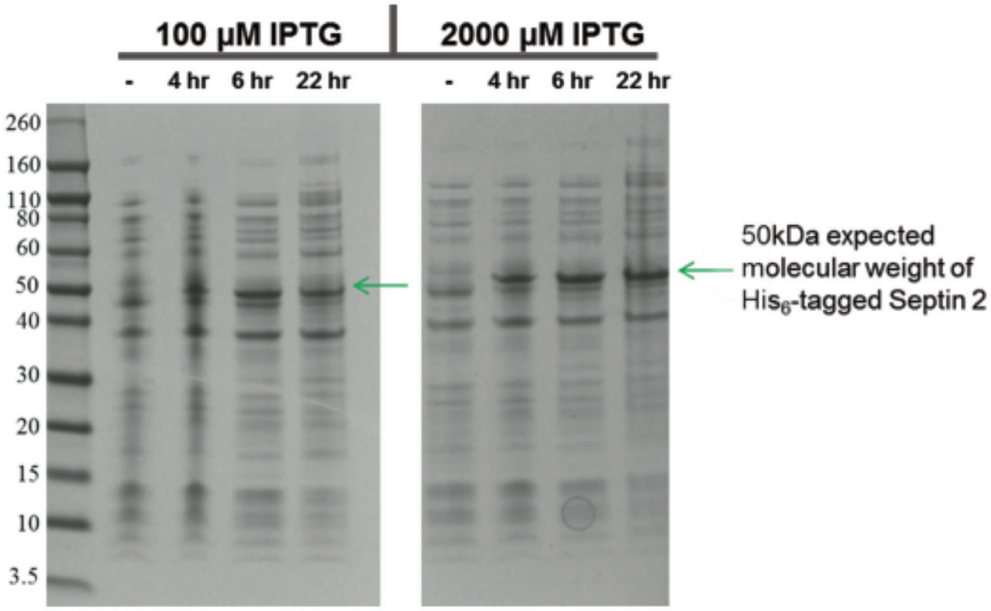

Figure 4. SDS-PAGE gels of total cell protein samples from bacteria transformed with a vector containing the Septin2 gene under the control of the P_{T7}-HIS_6 promoter. Samples were collected at different time points for two cultures before and after induction with two different concentrations of IPTG. In the presence of IPTG an induced protein of about 50 kDa can be seen which is absent in the uninduced sample (-).

Assembly		Bacterial Selection Maker	*E. coli* Origin of Replication	Expansion Position #1	Expansion Position #2	Promoter/Tag
1	**Negative Control**	Kan	p15A	XP1 Linker	LacI	P_{T7}-HIS
2	**Positive Control (lacZ†)**	Kan	p15A	XP1 Linker	LacI	P_{T7}-HIS
3	**Septin2†**	Kan	p15A	XP1 Linker	LacI	P_{T7}-HIS

†Gene-of-Interest (GOI)

Table 3. Three vector assemblies to test the functionality of the P_{T7}-HIS_6 promoter module in *E. coli*.

Functionality in Yeast

In order to demonstrate functionality in yeast, vectors were assembled using the yeast elements supplied in the SureVector Core Kit (yARS, LEU2 and the P_{GAL1}-HIS_6 promoter element). In addition to a negative control containing no GOI and a positive control containing lacZ, a gene encoding a green fluorescent protein (GFP) gene, was assembled into a vector construct (Table 4). The functionality of the LEU2 selectable marker and the P_{GAL1}-HIS_6 promoter were assessed after transformation into a leucine auxotrophic yeast strain. Yeast colonies transformed with the vector carrying the GFP gene were plated on synthetic medium that was devoid of leucine and either contained glucose or galactose as the sole carbon source (Figure 5). On the glucose medium (repressed) very little GFP could be observed. However, colonies grown on the galactose medium (induced) were significantly greener, indicating that expression of the GFP gene was strongly up-regulated, verifying that the P_{GAL1}-HIS_6 promoter was performing properly (glucose repressible and galactose inducible). On either medium plenty of LEU positive colonies were observed, demonstrating that the functional LEU2 selectable marker complemented the leucine auxotrophic marker.

In order to enable utility of the constructs in yeast systems, the yARS element must perform properly and enable accurate replication of the plasmid. To demonstrate full functionality of this module, plasmid DNA was recovered from individual yeast transformants (3-6 in Table 4) from four separate yeast transformations and then re-transformed into *E. coli.* Plasmid DNA from six of the subsequent bacterial transformants for each construct was subsequently isolated and digested with Xho I restriction enzyme. The banding pattern for all 24 plasmid isolates was identical to that of the input plasmid used to initially transform yeast, verifying the functionality of the yARS element and the fidelity of DNA replication in both yeast and bacteria (Figure 6).

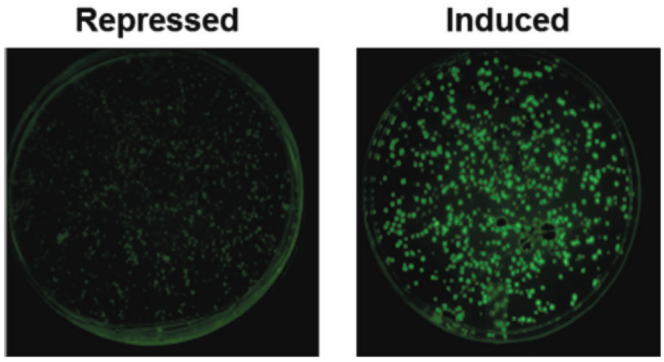

Figure 5. Transformants of a yeast leucine auxotroph using a vector containing the GFP and LEU2 genes. When plated on a complete synthetic medium (-leucine) with glucose as the carbon source, the promoter is repressed as expected, and the GFP gene is not expressed (left panel). When plated on a complete synthetic medium (-leucine) with galactose as the carbon source, the promoter is induced as expected and the GFP gene is expressed, resulting in green fluorescent colonies (right panel). Colonies on both plates indicate that the LEU2 selectable marker complements the leucine auxotrophic marker in the parental yeast strain.

Assembly		Bacterial Selection Maker	Origin of Replication	Expansion Position #1	Expansion Position #2	Promoter/ Tag Module
1	**Negative Control (No GOI)**	Kan	p15A	yARS	LEU2	P_{GAL1}-HIS
2	**Positive Control (lacZ†)**	Kan	p15A	yARS	LEU2	P_{GAL1}-HIS
3	**GFP† (green fluorescent protein)**	Kan	p15A	yARS	LEU2	P_{GAL1}-HIS
4	**RFP† (red fluorescent protein)**	Kan	p15A	yARS	LEU2	P_{GAL1}-HIS
5	**Test GOI A†**	Kan	p15A	yARS	LEU2	P_{GAL1}-HIS
6	**Test GOI B†**	Kan	p15A	yARS	LEU2	P_{GAL1}-HIS

†Gene-of-Interest (GOI)

Table 4. Four vector assemblies to test the yeast functional elements in the SureVector Core Kit.

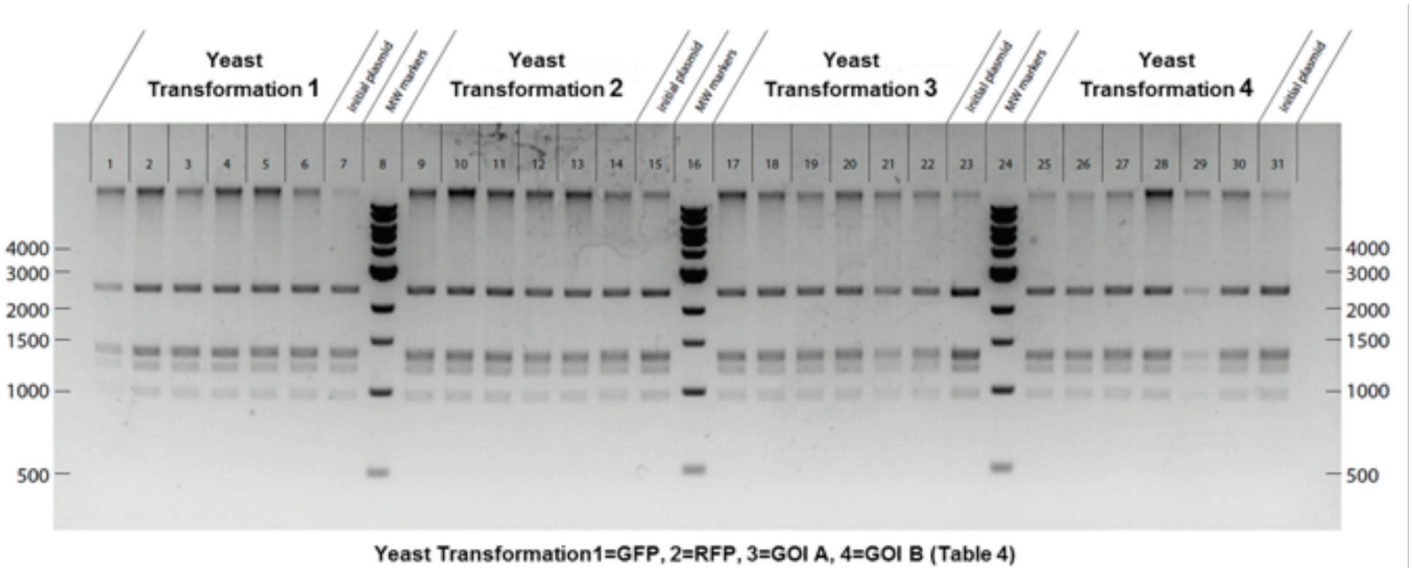

Figure 6. Xho I digest of plasmid DNA from six of the *E. coli* transformants for each construct. Plasmid DNA was recovered from each of the four separate yeast transformations (Table 4, 3-6) and re-transformed into *E. coli.* Plasmid DNA isolated from six colonies of each of the four bacterial transformants was then subjected to restriction enzyme digestion. The digestion patterns of all 24 bacterial transformants were identical to each other and the original plasmid construct, confirming that the yARS module correctly replicated the plasmids in yeast.

His-tag™ Purification of Expressed Proteins

The histidine tag sequence has been incorporated into all three promoter modules used in the SureVector Core Kit, in order to enable efficient purification of cloned proteins. To demonstrate that the tag works properly, cell extract was prepared from a GFP yeast transformant (Table 4). The extract was subjected to affinity chromatography using a nickel HisTrap column (Pharmacia) to purify the GFP.

Figure 7 shows the elution pattern of the GFP by the imidazole gradient, verifying that the His-tag is present and functional on the cloned GFP. The affinity column fractions from this purification, as well as one done with another expressed His-tagged red fluorescent protein (RFP, Table 4) also expressed in yeast, were further analyzed by gel electrophoresis (Figure 8). In both cases, protein of the correct size was obtained at fairly high purity, using the cloned His-tag for purification.

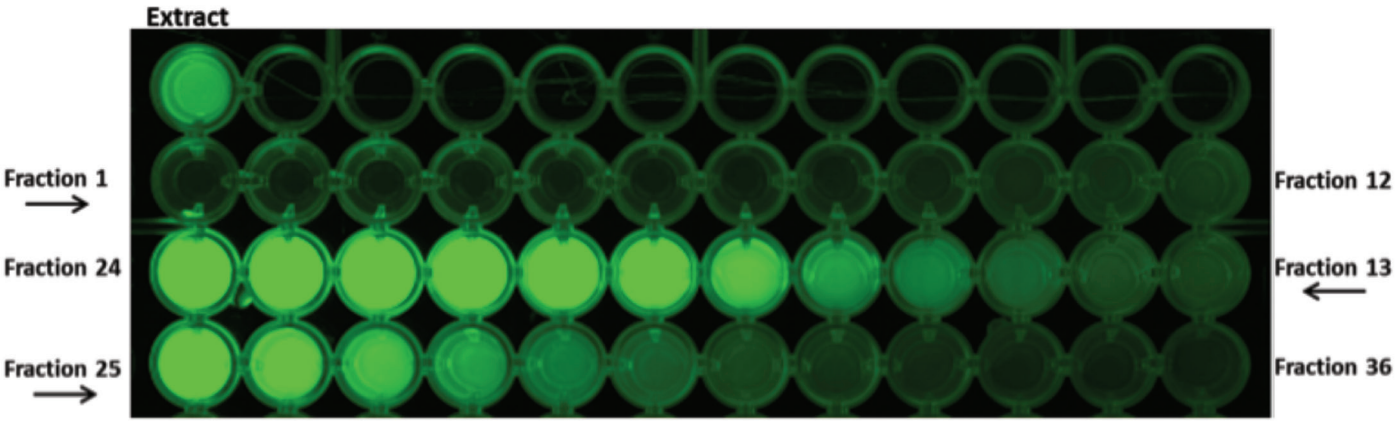

Figure 7. Affinity purification of a His-tagged GFP yeast transformant cell extract using a nickel HisTrap column. An imidazole gradient was used, starting with Fraction 1, to elute the bound His-tagged GFP from the column, demonstrating that the HIS_6 tag incorporated into the SureVector is functional. The crude yeast extract before purification is shown in the upper left corner. The GFP protein was visualized using a fluorescent light source.

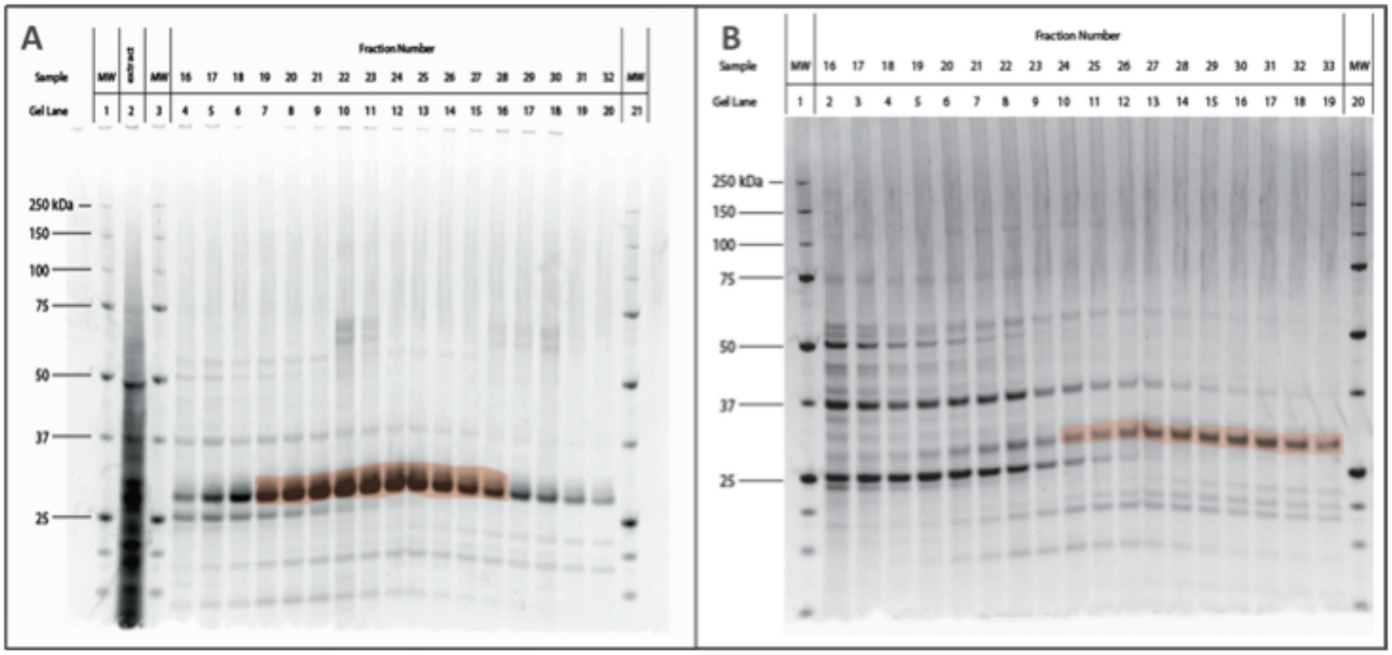

Figure 8. Gel electrophoretic analysis of His-tagged GFP (A) and his-tagged RFP (B) elution fractions obtained using His-tag affinity chromatography. In each case, the purification provided fairly pure protein of the correct size (highlighted fractions); validating the expression and proper function of the HIS_6 tag incorporated into the SureVector promoter modules.

Functionality in Mammalian Cells

Two different vector assemblies containing the neomycin resistance marker were constructed and sequence verified. One vector also contained the ampicillin resistance marker and the CMV-His promoter fused to GFP, while the second vector contained the chloramphenicol resistance marker and the CMV promoter fused to GFP. Both plasmids were transfected **into a human cell line** and subjected to selection using a neomycin analogue at a final concentration of 500 µg/mL.

The transfected cells exhibited robust growth and good GFP expression in all surviving cells (+25 days), as shown in Figure 9. Negative control cells (i.e., untransfected cells) all died within a few days, and positive control cells (i.e. transfected cells with a plasmid containing the neomycin resistance marker) showed robust growth similar to control transfections. Both plasmids conferred on the cells the ability to survive in the presence of a neomycin analogue at a toxic concentration for over 25 days, producing stable cell lines expressing GFP.

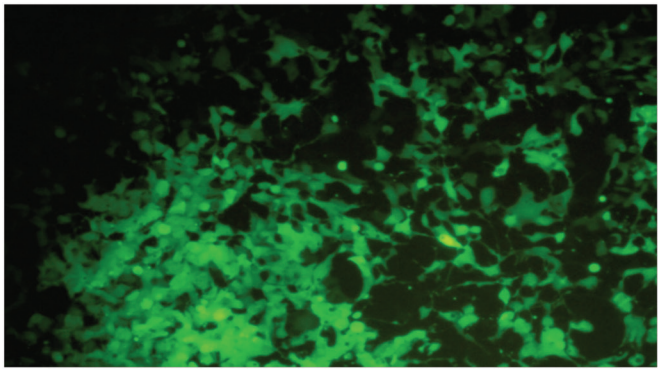

Figure 9. Image of mammalian cells transfected with a SureVector containing the neomycin resistance gene after 31 days of growth on toxic media.

Summary

SureVector is the only next-generation plasmid assembly technology that provides validated and guaranteed assembly of multiple functional DNA fragments into vectors for use in bacterial, yeast and mammalian systems. Using a single 20 minute reaction, a custom vector containing one of 216 possible configurations of standard DNA modules can be generated that will provide efficient and reliable transformation and expression in the system of choice. This unparalleled level of performance has been assured by extensive validation using DNA sequencing, restriction enzyme analysis of plasmid constructs after propagation in multiple systems, expression of a variety of genes of interest in multiple systems, and affinity purification of expressed proteins.

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