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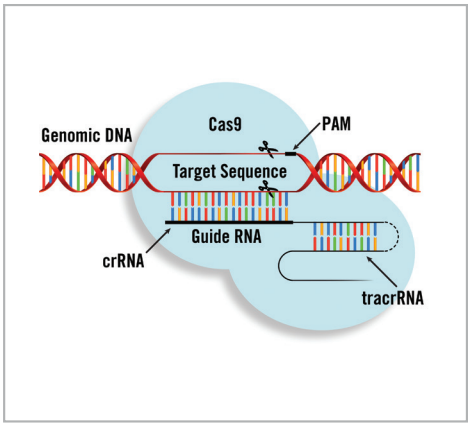

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Superior Performance of Omega Bio-tek's E. Z. N. A.® Soil DNA Kit over Company M's Soil DNA Isolation Kit for DNA Extraction from Soil Samples

By Kiranmai Durvasula, Julie Baggs, Travis Butts, Omega Bio-tek, Inc, Norcross GA 30071

INTRODUCTION

Molecular analysis of soil DNA offers a direct solution for detecting microorganisms residing in soil and studying microbial diversity. Isolation of DNA from soils is often challenging because of the presence of many contaminants, such as humic acid, that can interfere with the extraction process and inhibit several downstream applications. An ideal DNA extraction method should effectively eliminate inhibitory substances and maximize DNA yields. The main objective of this study was to compare the performance of Omega Bio-tek's E. Z. N. A. Soil DNA kit (101319-090) to that of Company M's Soil DNA Isolation kit in terms of DNA yield and quality, as well as amplification potential and detection sensitivity using real-time PCR.

MATERIALS AND METHODS

Total DNA was isolated from 200mg of outdoor soil spiked with 10µL of ZymoBIOMICS™ Microbial Community Standard (Zymo Research) using E. Z. N. A. Soil DNA kit (Omega Bio-tek) and Company M's Soil DNA Isolation kit. ZymoBIOMICS Microbial Community Standard is comprised of ten microbial strains — three easy-to-lyse Gram-negative bacteria, five tough-to-lyse Gram-positive bacteria, and two tough-to-lyse yeasts. In this experiment, this mix serves as a benchmark for performance comparison of the two kits. The soil samples were homogenized using the Omni Bead Ruptor 24 and the isolations were done in triplicate following manufacturer's recommended protocols for each kit. The protocol times were approximately 60 minutes and 70 minutes for Company M and Omega extractions respectively, and the ease of use was comparable. Purified DNA was eluted in 100µL of each kit's elution buffer and quantified using Promega QuantiFluor® dsDNA system. The quality of the purified DNA from both the kits was analyzed by performing real-time PCR using 16S bacterial specific primers on 10X, 100X, 1000X dilutions of the purified DNA. It was further tested for relative abundance of two tough-to-lyse strains of *Listeria monocytogenes* (Gram-positive bacteria) and *Saccharomyces cerevisiae* (yeast) on 10X and 100X dilutions employing either *Listeria* specific primers or *Saccharomyces* specific primers. Briefly, a qPCR reaction was set up to a total volume of 20µL using Agilent's Brilliant III 2X SYBR® as the master mix and 2µL of template DNA at appropriate dilutions amplified with suitable primers following a standard protocol on the ABI 7900.

RESULTS AND DISCUSSION

The DNA yields from the soil samples spiked with ZymoBIOMICS using the Omega kit and Company M kit are as shown in Figure 1. The performance of the Omega kit was significantly better compared to Company M's with a 40% increase in yield when eluted in 100µL final volume (8.1µg vs 5.7µg) ($p<0.05$; *Tukey's post-hoc* analysis).

The quality of the DNA obtained from each extraction was determined based on the C_t values generated from a qPCR reaction. Table 1 shows the average C_t values obtained on serial dilutions of the purified DNA using 16S bacterial specific primers. The C_t's seem to be slightly lower (~0.5) with the Omega extractions. Figure 2 shows average C_t values obtained using *Listeria* and *Saccharomyces* specific primers using 10X and 100X diluted purified DNA as the template. The organisms (*Listeria monocytogenes* and *Saccharomyces cerevisiae*) investigated here are both tough-to-lyse and the results demonstrate the C_t's with Omega kit were significantly lower than Company M's ($p<0.05$; *Tukey's post-hoc* analysis). C_t's were lower by one cycle for the Gram-positive bacterium, *Listeria* and almost three cycles lower for the yeast strain, *Saccharomyces*; that is, a two-fold and eight-fold higher yield of *Listeria* and *Saccharomyces* was obtained with the Omega kit when compared to the Company M kit. The ΔC_t between the serial dilutions was comparable for both the kits (~3.1 for *Listeria* and ~3.4 for

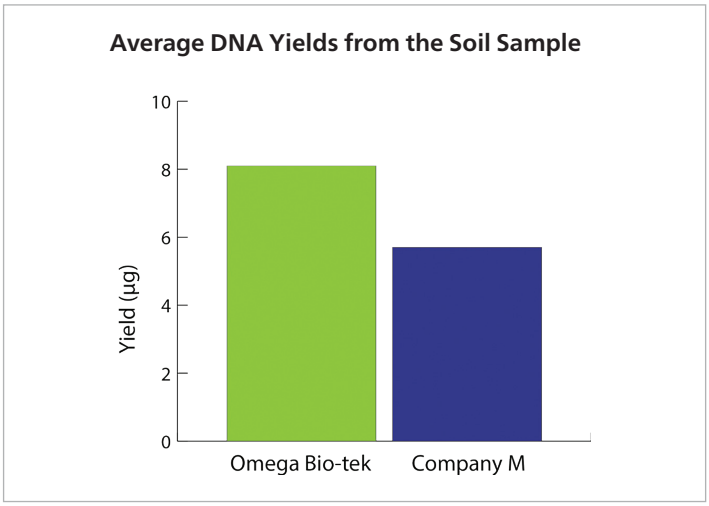

Figure 1. Average DNA yields from both the kits using Omni Bead Ruptor 24 as the homogenizer (*p < 0.05).

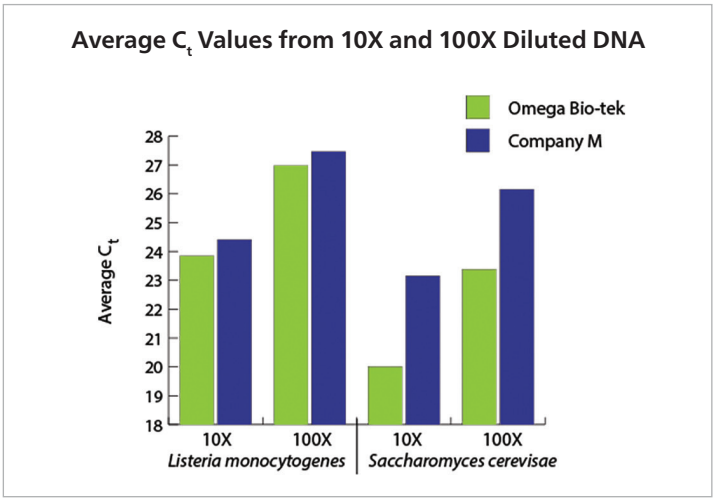

Figure 2. Average C_t values obtained after amplifying the purified DNA from Omega and Company M kits with *Listeria* and *Saccharomyces* specific primers.

	C_t		
	10X	**100X**	**1000X**
Omega Bio-tek	14.93	17.42	21.46
Company M	15.34	18.22	21.97

Table 1. Average C_t values from 10X, 100X, 1000X dilutions of purified DNA using Omega and Company M kits using 16S bacterial specific primers.

Saccharomyces). This data suggests that Omega Bio-tek's kit performed as well as the Company M kit in eliminating PCR inhibitors from the isolations but with superior yields. The C_t values obtained on qPCR corroborate with the yields obtained, with Omega Bio-tek's E.Z.N.A. Soil DNA Kit exceling on both fronts.

CONCLUSIONS

The Omega Bio-tek kit isolated DNA with significantly higher yields and was better able to isolate tough-to-lyse organisms than the Company M kit for the soil sample tested. The Omega kit effectively removed the PCR inhibitors exemplified by the fact that the Omega-isolated DNA amplified consistently sooner than the Company M-isolated DNA, which is represented by the lower C_t values. The lower C_t values may also indicate higher quality of the eluted DNA obtained using the Omega Bio-tek's kit. Overall, the results show that the Omega Bio-tek's E.Z.N.A. Soil DNA Kit isolates high yielding, high quality DNA compatible with various downstream applications such as qPCR and next-generation sequencing (NGS).

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Cycles	1–10

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Biometra TOne 96G, 230 V	**75873-992**	**Each**

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Substrate Specificity and Mismatch Discrimination in DNA Ligases

by Greg Lohman, Ph.D., New England Biolabs, Inc.

DNA ligases are enzymes that seal breaks in DNA by joining 5´-phosphorylated DNA termini to 3´-OH DNA termini[1-4]. *In vitro,* ligases (notably T4 DNA Ligase) are critical reagents for many molecular biology protocols, including vector-insert joining for recombinant plasmid construction, adaptor ligation for next-generation sequencing (NGS) library construction, and circularization of dsDNA[6]. Less commonly utilized *in vitro, Taq* DNA Ligase will ligate only nicks[5-8]. *Taq* ligase is a NAD^+-dependent DNA ligase from a thermostable bacterium that can survive high temperatures (up to 95°C) and is active over a range of elevated temperatures (37–75°C).

T4 DNA Ligase will efficiently ligate many undesirable structures, including substrates containing gaps of one or more nucleotides and nicked substrates that contain DNA base pair mismatches[8-11]. For some applications, however, there cannot be any end-joining activity at all, and for others, there is a need for the exclusive ligation of fully base-paired nicks with no gaps. For example, DNA assembly methods, such as Gibson Assembly® and NEBuilder® HiFi DNA Assembly, require nick-selective ligases, such as *Taq* DNA Ligase, which only reacts with substrates containing no gaps, and will not join any fragments end-to-end without the exo/polymerase generation of annealed complementary regions.

Numerous applications have been developed that take advantage of the high fidelity of *Taq* and other thermostable DNA ligases to detect specific nucleotide sequences with high specificity and quantitative accuracy, including profiling single nucleotide polymorphisms (SNPs)[5,12,13]. In the Ligase Detection Reaction (LDR), a set of probes complementary to the sequence of interest are annealed to target DNA (genomic DNA or a PCR amplified fragment) and treated with a high-fidelity thermostable DNA ligase. If the target sequence is present, the probes will ligate; cycling through rounds of melting and annealing can allow linear amplification of the probe ligation product. With the right ligase, conditions and suitable probes, single-base differences can be reliably detected. The original paper detected the ligation product through visualization in a gel, but detection through fluorophore-quencher pairs or qPCR-based methods can greatly increase the sensitivity of detection[14-20]. LDR has also been extended to multiplexed probe sets that allow the simultaneous interrogation of multiple potential SNP sites[15].

Ligase Specificity

DNA ligases generally prefer fully Watson-Crick base-paired dsDNA substrates to those containing one or more mismatches. However, ligases can ligate some mismatches to a significant degree, and very active ligases, such as T4 DNA Ligase, can ligate nicks containing one or more mismatches near the ligation junction with high efficiency[11,21]. Ligases are thought to interrogate dsDNA for proper base pairing through minor grove contacts, and thus do not read specific base sequences, but are sensitive to distortions in helix shape[22]. Large purine:purine mismatches and most smaller pyrimidine:pyrimidine mismatches are typically worse ligation substrates than pyrimidine: purine mismatches. Helix stability also plays some role, and mismatches with more hydrogen bonds are more readily ligated than those with few. For many ligases, G:T mismatches with two hydrogen bonds and a base-pair size that is nearly indistinguishable from a Watson-Crick base pair are joined with nearly the same efficiency as a correct base pair. Additionally, DNA ligases have been generally found to have a higher discrimination at the upstream side of the ligation junction (the base pair providing the 3´-OH terminus to the ligation) than on the downstream side (the base pair providing the 5´-phosphate to the ligation). The structural/mechanistic reason for this differential is not known for certain but may have to do with the slight melting of the 5´-terminus during the reaction. This "peeling back" of the 5´-phosphorylated base can be observed in the crystal structures of several DNA ligases bound to substrate[23,24].

Thermostable DNA ligases, including *Taq* DNA Ligase, are naturally able to discriminate against ligating substrates containing base pair mismatches (i.e., are "higher fidelity") than T4 DNA Ligase[21,25,26]. Despite this higher fidelity, *Taq* DNA Ligase can still detectably ligate many T:G, T:T, and A:C mismatches. Thermostable DNA ligases are active at elevated temperatures, allowing further discrimination by incubating the ligation at a temperature near the melting temperature (T_m) of the DNA strands. This selectively reduces the concentration of annealed mismatched substrates (expected to have a slightly lower T_m around the mismatch) over annealed fully base-paired substrates. Thus, high-fidelity ligation can be achieved through a combination of the intrinsic selectivity of the ligase active site and careful balance of reaction conditions to reduce the incidence of annealed mismatched dsDNA.

Optimization Through High-Throughput Profiling

NEB® researchers recently published a method for the high-throughput profiling of ligase fidelity, a method that extends earlier studies through a high-sensitivity multiplexed format[21,27]. This methodology

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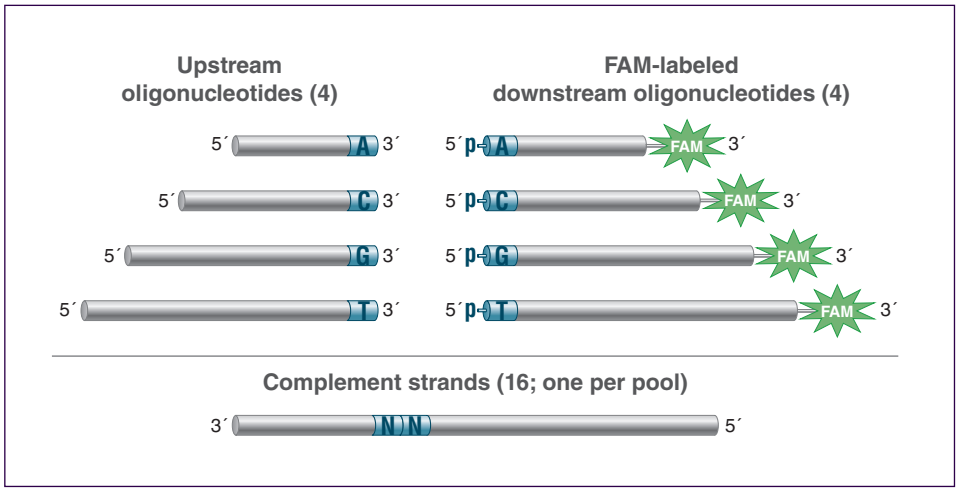

Figure 1. Schematic of multiplexed substrate pools Each substrate pool contained a single splint with a defined NN at the ligation junction (e.g., AA, AC, AG…) along with all four upstream probes and all four FAM-labeled downstream probes. Each probe is a unique length that encodes the base at the ligation junction: 20, 28, 36 and 44 bases for the 3´-A, C, G and T´ terminated upstream probes; 30, 32, 34 and 36 bases for the 5´-pA, pC, pG, and pT terminated, 3´-FAM-la-beled downstream probes. A total of 16 substrate pools were prepared, one for each unique splint. Image adapted from Lohman, G.J. et al. 2016.

has been used to rapidly screen buffer conditions for *Taq* DNA Ligase and their effect on fidelity. In this method, substrate pools were prepared consisting of one target (template) strand and four upstream probes and four down-stream probes, each differing only in the base at the ligation junction. Thus, all four bases at either side of the ligation junction were represented. Sixteen separate pools were prepared, each with a different template strand covering all 16 possible NN pairs in the template as well. The probes were designed such that each possible pairing resulted in a product of unique length, with products repeatable and quantifiable by capillary electrophoresis (CE) (Figure 1). This method allowed screening of all possible base combinations (Watson-Crick and mismatched) around the ligation junction in 16 wells of a 96-well plate, allowing six conditions to be screened per plate. The results indicated that the optimal buffers for high fidelity ligation by *Taq* DNA Ligase contain 100–200mM KCl at pH 8.5.

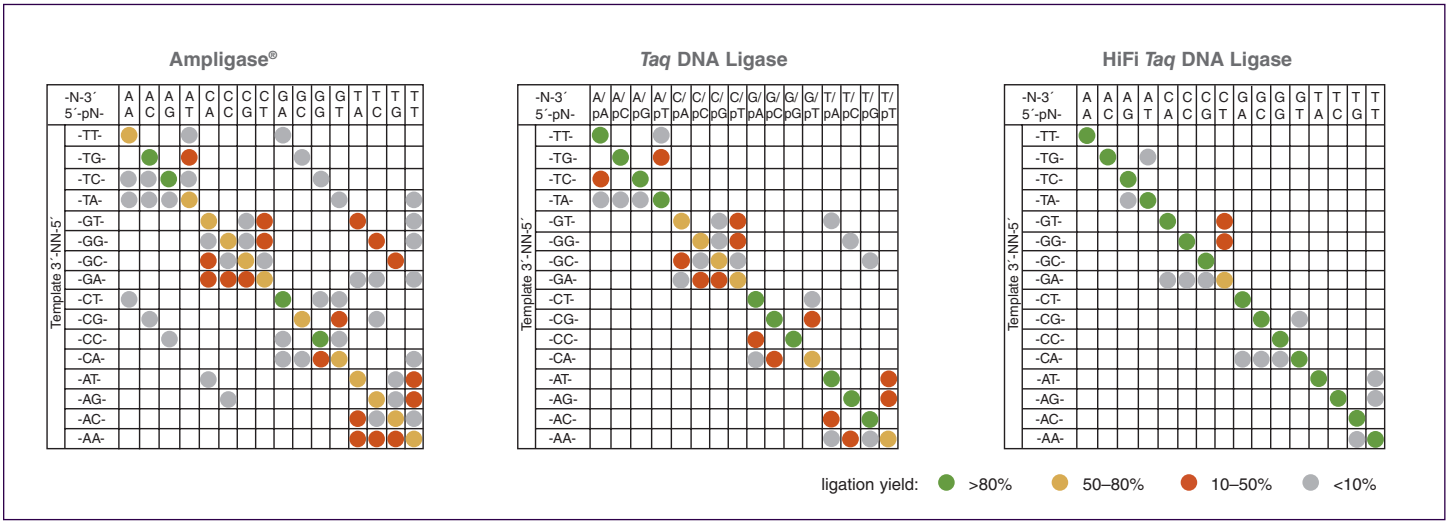

Figure 2. Comparison of fidelity of *Taq* DNA Ligase (NEB), AmpLigase (Epicentre), and HiFi *Taq* DNA Ligase (NEB) Fidelity measurements were performed using 1µL of ligase in a 50µL reaction mixture in the supplied buffers at 1X concentration. Reactions were incubated 30 min at 55°C, using multiplexed substrate pools as outlined in our previous publication (20). The rows represent a single template sequence, while columns indicate a particular ligation product resulting from a specific pair of probes ligating with the indicated bases at the ligation junction. A dot indicates detection of a product (see legend). The diagonal from the top left to the bottom right represents Watson-Crick ligation products; all other spaces indicate mismatch ligation products. While *Taq* ligase and Ampligase perform similarly under these conditions, with a range of mismatch products detectable, HiFi *Taq* Ligase shows dramatically fewer mismatch products while maintaining high yields. Image adapted from Lohman, G.J. et al. 2016.

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This optimization method has been used internally at NEB to screen additional ligases, conditions, and formulations and has led to the development of the new HiFi *Taq* DNA Ligase. Using this method, both the enzyme and the reaction buffer were optimized, resulting in the highest fidelity NAD^+-dependent DNA ligase commercially available (Figure 2).

Conclusion

It is important to note that thermostable, high-fidelity, nick-selective DNA ligases like *Taq* DNA Ligase, HiFi *Taq* DNA Ligase, and the ATP dependent 9°N™ DNA Ligase are not replacements for T4 DNA Ligase in applications such as routine cloning or DNA library preparation. However, when a method relies on accurate ligation of nicks lacking gaps or mismatched base pairs (as in the case for LCR, LDR, and Padlock Probes), using of one of these ligases, combined with careful probe design and reaction condition optimization, will be critical for success.

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Standard Ready PCR Mix, 2X and Lyophilized Format

INTRODUCTION

Ready PCR Mix is a convenient master mix that facilitates efficient sample preparation for both standard amplification reactions and high-throughput colony PCR. The mix contains high fidelity Extender™ DNA Polymerase Blend, reaction buffer, and dNTPs, as well as electrophoresis tracking dye and non-mutagenic EZ-Vision® DNA dye. The single red tracking dye allows for direct-to-gel loading of PCR amplicons and the EZ-Vision DNA dye stains the samples during electrophoresis, so that post-run, the DNA may be viewed immediately without further staining or destaining. Ready PCR Mix is available in standard 2X solution format and as a lyophilized powder for extended storage.

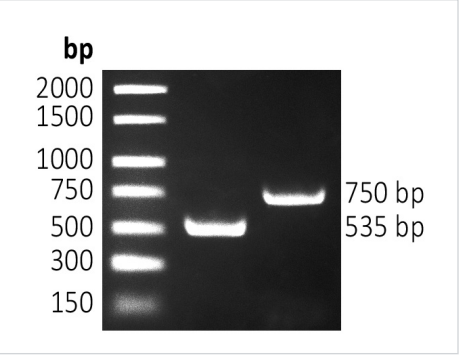


Figure 1. Efficient amplification of purified DNA with Ready PCR Mix, 2X. Single copy targets were amplified from 50ng of pUC19 plasmid (535 bp) or *S. aureus* genomic DNA (750bp) for 35 cycles. Aliquots (10µL) of each PCR reaction were directly loaded onto a 1% agarose gel with PCR DNA Marker and visualized with UV transillumination and a green filter.

MATERIALS AND METHODS

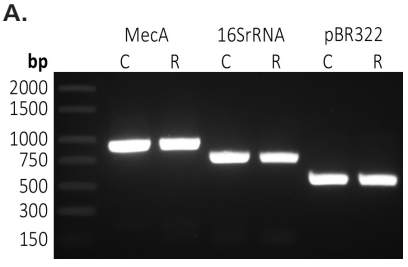
Standard PCR and Direct-to-Gel Loading with Ready PCR Mix

Ready PCR Mix is a 2X master mix designed to simplify reaction preparation upstream of thermal cycling and minimize sample handling after amplification. A test of the mix in standard PCR was initiated by addition of an equal volume of Ready PCR Mix to tubes containing 50ng of S. aureus genomic DNA or pUC19 premixed with their respective primers at 1.5µM each. Following 35 amplification cycles, 10µL of PCR products were loaded directly from the PCR tubes onto a 1% agarose gel with TAE buffer and resolved. Post-electrophoresis, the gel was immediately placed on a UV transilluminator and an image of the amplicons and marker was captured using a green filter (500–600nm). Distinct bands of 535bp and 750bp were observed as expected from the *S. aureus* and pUC19 reactions, respectively, demonstrating the utility of Ready PCR Mix as a master mix (Figure 1).

Comparison of Control and Ready PCR Reactions

Since Ready PCR Mix contains both tracking and DNA dye, concern that there may be considerable inhibition of amplification needed to be addressed. A comparison of PCR reactions setup using Extender DNA Polymerase Blend and Ready PCR Mix was performed to quantitate relative amplicon yields by gel. DNA fragments were amplified with three different primer sets in reactions containing either 50ng *S. aureus* genomic DNA or pUC19 plasmid DNA. Following simultaneous amplification in a standard thermal cycler, the amplicons from *S. aureus mecA* and *16S rRNA* genes, as well as DNA from pUC19 were mixed with EZ-Vision One DNA Dye as Loading Buffer, 6X and resolved on a 1% agarose gel with TAE buffer (Figure 2A). Samples derived from control reactions using Extender DNA Polymerase Blend (Figure 2A, C) and from Ready PCR Mix reactions (Figure 2A, R) were run in adjacent lanes and their integrated band densities compared. The Ready PCR products were present at 96.5% (mecA), 87.4% (16S rRNA) and 94.4% (pUC19) relative to the control products (Figure 1). A mild reduction in amplicon yield in Ready PCR

A.



B.

Primers	Band Densities: Extender™ Control Reaction	Ready PCR Mix Reaction	Ready PCR Mix Normalized to Control
MecA	738116	712077	96.5%
16S rRNA	575219	619312	87.4%
pBR322	619312	584907	94.4%

Figure 2. Amplicon yield by gel in Ready PCR reactions normalized to control reactions. Single copy targets were amplified from 50ng of *S. aureus* genomic DNA (MecA, 16S rRNA) or pUC19 plasmid (pBR322) in control reactions prepared with Extender DNA Polymerase Blend (C) and with Ready PCR Mix (R). Aliquots (10µL) of each PCR reaction were directly loaded onto a 1% agarose gel and visualized with UV transillumination and a green filter (A). Integrated band densities from the gel image for Ready PCR Mix were normalized to the control bands (B) with the MecA,16S rRNA and pBR322 amplicons present at 96.5%, 87.4%, and 94.4% of the controls, respectively.

reactions due to slight inhibitory effects of the included tracking and DNA dyes was noted. This did not affect detection of the amplicons in Ready PCR Mix samples.

Sensitivity of Ready PCR Mix

In order to determine the sensitivity of the all-in-one master mix, a ten-fold dilution series of pUC19 template DNA from 10ng to 1pg was used in PCR reactions with the reconstituted lyophilized version of Ready PCR Mix. Following 30 amplification cycles of single-copy target DNA, 10µL of the reactions were loaded directly onto a 1% agarose gel and resolved in TAE buffer. Visualization of the gel by UV transillumination and a green filter (500–600nm) revealed the sensitivity of Ready PCR Mix goes down to 1pg of DNA template (Figure 3).

Efficient Colony Screening with Ready PCR Mix

Colony screening, a method in which transformed bacteria containing a desired insert are quickly identified by PCR, was tested with Ready PCR Mix. Single, well-isolated colonies of *E. coli* transformed with pUC19 containing yeast Ded1 were picked and directly resuspended into separate tubes of Ready PCR Mix premixed with primers. A 5µL volume from each PCR reaction was transferred to LB medium with ampicillin and immediately incubated at 37°C for growth, while remaining reaction volumes were placed in a thermal cycler for amplification. Positive colonies were identified by directly loading the PCR products onto a gel and imaging the DNA post-electrophoresis without any additional staining or destaining steps (Figure 4). The corresponding starter cultures were ready for inoculation of overnight cultures within a few hours of completion of amplification.

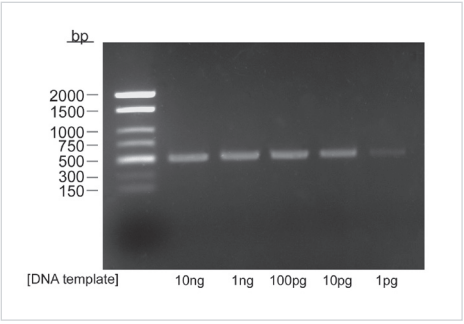


Figure 3. Sensitivity of Ready PCR Mix. Ten-fold serial dilutions of pUC19 were added as templates to reactions prepared using reconstituted Lyophilized Ready PCR Mix. Target DNA was amplified for 30 cycles and afterward a volume of 10µL of per reaction was directly loaded onto a 1% agarose gel and resolved along with PCR DNA Marker mixed with EZ-Vision® One DNA Dye. The gel was visualized with UV transillumination and a green filter.

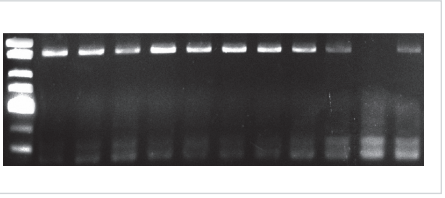


Figure 4. Efficient colony screening with Ready PCR Mix. Single colonies of transformed pUC19 were resuspended into Ready PCR Mix premixed with target primers. A 5µL volume of the reaction was transferred to selective LB medium for growth while the remaining volume was amplified for 30 cycles. Following amplification, 10µL of each PCR reaction were directly loaded onto a 1% agarose gel and visualized with UV transillumination and a green filter.

CONCLUSIONS

PCR master mixes are popular for good reason. Comprised of all the necessary reaction components in a single tube, except for experiment-specific template and primers, they minimize the number of pipetting steps and consequential pipetting errors, as well as the chance for contamination. Ready PCR Mix offers the benefits of a master mix and is further enhanced by the addition of both a fast-migrating tracking dye (10bp on a 1% agarose gel) and non-mutagenic EZ-Vision DNA dye.

The magenta tracking dye allows for direct loading and monitoring migration of Ready PCR reactions on an agarose gel following amplification. After electrophoresis, the included EZ-Vision DNA dye facilitates the immediate visualization of PCR products using standard UV illumination. No post-run staining and destaining steps are necessary. As little as 1pg of DNA template can be amplified and detected using Ready PCR Mix, which is available in standard solution and lyophilized formats. Ready PCR Mix is stable for at least 15 freeze-thaw cycles and is compatible with standard downstream applications, such as PCR purification. Ready PCR Mix is ideal for high-throughput PCR, including colony screening, a method that simplifies molecular cloning.

Description	Size	Cat. No.	Unit
Ready PCR Mix, 2X	1.25 mL	**97063-320**	**Each**
Ready PCR Mix, 2X	2 x 1.25 mL	**97063-322**	**Each**
Lyophilized Ready PCR Mix, 2X	2 Vials	**97068-170**	**Each**

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- Certified for shipping

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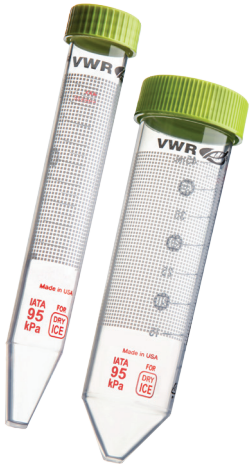

Description	Package	Cat. No.	Pk/Cs
15mL CO_2 Resistant Tube, Non-Sterile	Sleeves of Ten	10852-306	50/100
15mL CO_2 Resistant Tube, Sterile	Sleeves of Ten	10852-302	50/100
50mL CO_2 Resistant Tube, Non-Sterile	Sleeves of Ten	10852-308	50/100
50mL CO_2 Resistant Tube, Sterile	Sleeves of Ten	10852-304	50/100

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Low Retention Tips Performance Comparison

ABSTRACT

Pipetting accuracy and precision are the key factors for successful experiments in the laboratory. The best pipetting performance is achieved, when the pipette and the tip match perfectly with each other, and the tip being used is optimal for the liquid being transferred.

When pipetting liquids with low surface tension (e.g., detergents), a film of liquid is often left on the inner surface of standard polypropylene pipette tips, leading to inconsistency and inaccuracy in pipetting, as well as loss in volume of valuable samples.

Various technologies are being used to modify the standard pipette tips to mitigate this issue. This application note describes the comparison of various manufacturers' low retention tips when handling liquids that contain detergents.

The results show that Sartorius Low Retention Tips ensure near complete recovery of the sample when pipetting liquids of low surface tension. When compared to other low retention tips from various manufacturers, Sartorius tips secured the best sample recovery and the best chemical resistance.

INTRODUCTION

In many molecular biology applications the increased sensitivity of the detection methods call for extreme reliability and reproducibility in pipetting. In DNA and protein analysis methods the reagents and/or the samples often contain detergents. Pipetting liquids that contain detergents can be problematic when using standard pipette tips. Often residue of the liquid remains in the tip due to differences in surface energies between the sample and the plastic pipette tip. This, sometimes invisible, film on the tip's inner surface causes imprecision in pipetting and a loss of valuable samples or expensive reagents. Sartorius has utilized an advanced technology to create an extremely hydrophobic yet durable surface on their Low Retention Tips. This feature helps users to clearly reduce the amount of residue in the tip when handling detergents or other liquids that have a low surface tension. Better reproducibility in pipetting is especially beneficial in sensitive applications like PCR, or real-time PCR.

In this application note we:

1. Compare various pipette tips with a low retention feature, which are usually used when pipetting common detergent solutions found in molecular biology laboratories.
2. Show the results of a test where the chemical resistances of various manufacturers' low retention tips have been compared.

MATERIALS AND METHODS

Materials

- Sartorius Optift and SafetySpace standard and Low Retention Tips: 120µL, 200µL, 350µL, 1000µL
- Sartorius Picus® electronic pipettes: 5-120µL, 10-300µL, 50-1000µL, aspirating at speed 4, dispensing at slowest speed 1
- Sartorius mLINE® mechanical pipettes: 20-200µL, 100-1000µL
- Detergent solutions: 0.1% Triton™ X-100, 10% Tween® 20, 10% SDS, Colored 10X PCR buffer (contains detergents, density reagent, and tracking dyes)
- Other chemicals: Isopropanol, Acetonitrile, DMF (dimethylformamide)
- Green food dye
- Low retention pipette tips from five other manufacturers
- Sartorius MC5 Microbalance, Sartorius Analytical Balance BP211D
- Spectrophotometer for 96-well microplates (Biotek)

sartorius

Gravimetric Method

The liquid residue remaining in the pipette tip after dispensing was measured using the gravimetric method. A small glass container filled with a test solution was placed on the balance and the weight was set to zero. A desired volume of the test solution was then aspirated from the container and dispensed back. The balance reading, indicating the amount of liquid that remained in the tip, was then recorded. This was repeated for each detergent solution listed above.

Absorbance Test

In the absorbance test a colored test solution (green food dye dissolved in distilled water) was used to determine the residual liquid in the pipette tips after dispensing. The maximum nominal volume of the tested tip was used to aspirate the green test solution. The liquid was then dispensed directly back into its container. The tip was then rinsed five times with distilled water using the maximum volume of the tip. The absorbance of this solution was then measured using a spectrophotometer (405nm), and the results were compared to the reference solution. The absorbance of the tip rinsing solution directly correlates to the amount of residue in the tip.

Test for Chemical Resistance

1000µL of three solvents — isopropanol, acetonitrile and dimethylformamide — was aspirated and dispensed 20 times each with a 1000µL pipette tip. Afterwards, the tips were rinsed three times with distilled water. The effect of this treatment on the performance of the selected low retention tips was analyzed with the absorbance test using colored liquid as a test solution. The test was repeated with six tips for every solvent. The results of the chemically treated tips were compared to untreated standard tips and untreated low retention tips.

RESULTS

Comparison of Residual Liquid Volumes

The performance of five other manufacturers' low retention tips were compared with Sartorius' standard and Low Retention Tips, when pipetting commonly used detergent solutions. With all the liquids tested, Sartorius Low Retention Tips retained the least amount of residue (Figure 1a). Some of the competitors' low retention tips performed worse than even Sartorius' standard tips, suggesting that there are significant differences in performance among the low retention tips available on the market.

In another experiment a colored detergent-containing PCR buffer was used as a test solution to compare pipetting precision of various low retention tips and to see how the pipetting volume affects liquid retention in the tip. As shown in Figure 1b, Sartorius Low Retention Tips retained the

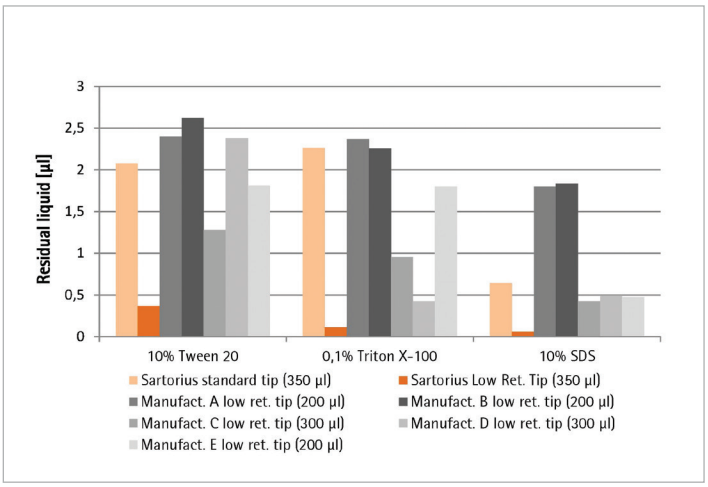

Figure 1a. Comparison of residual liquid amounts. Low retention tips from five manufacturers were compared with Sartorius standard and Low Retention Tips, when pipetting 200µL of the following detergents: 10% Tween 20, 0,1% Triton X-100 and 10% SDS. Pipette tips of volumes 200µL, 300µL, and 350µL (size depending on the manufacturers' offering and compatibility) were used with Picus® electronic pipette 10–300µL. The remaining liquid amount in the tip was measured using the gravimetric analysis method described in the Materials and methods. The test was repeated for 10 tips of each manufacturer.

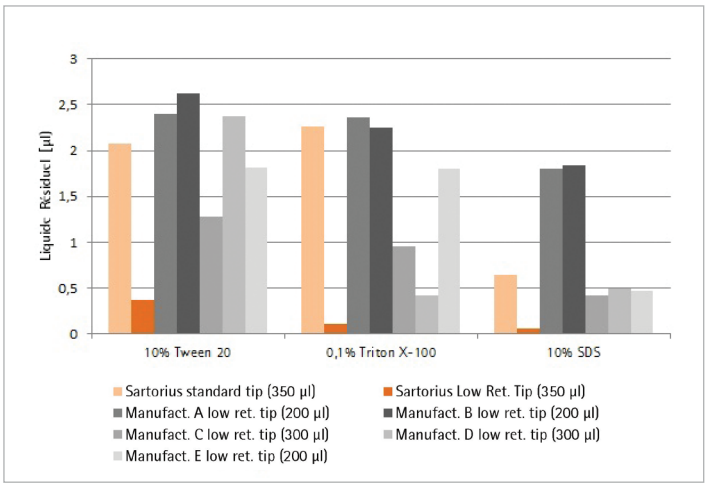

Figure 1b. Comparison of residual liquid amounts and pipetting precision. A coloured PCR buffer was used in 2 volumes. Low retention tips from five other manufacturers were compared with Sartorius standard and Low Retention Tips (filtered and non-filtered). Pipette tips of volume 120µL, 200µL and 300µL (size depending on each manufacturers' offering and compatibility) were used with Sartorius mLINE® 20–200µL to aspirate and dispense both 20µL and 120µL of a colored PCR buffer. The remaining liquid amount in the tip was measured using the gravimetric analysis method described in Materials and Methods. The test was repeated for 10 tips of each manufacturer. The error bars show the standard deviations.

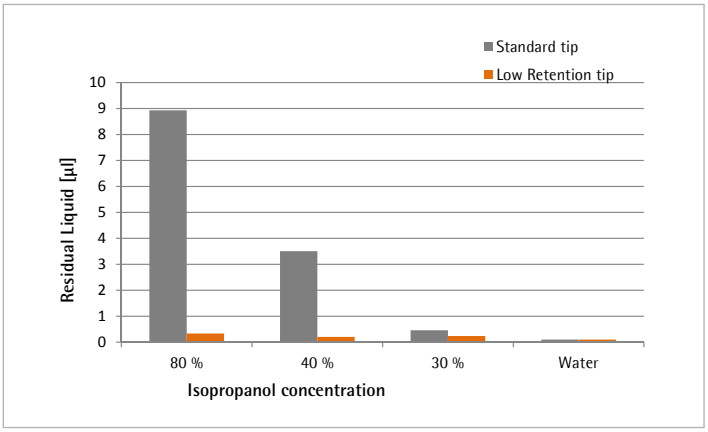

Figure 2. Effect of liquid surface tension to the amount of residue in the tip. 1000µL of various concentrations of isopropanol (30%, 40%, 80%) and distilled water were aspirated and dispensed with both Sartorius standard and Low Retention Optifit Tips (1000µL) using Sartorius Picus® electronic pipette (1000µL). The residual liquid in the tips was measured using the gravimetric analysis method described in the Materials and methods. The test was repeated for 10 tips of each manufacturer.

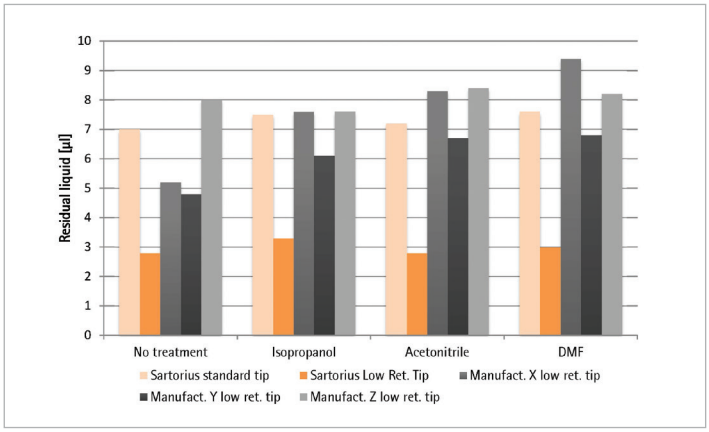

Figure 3. Comparing the chemical resistance of low retention pipette tips Low retention tips from three manufacturers were compared with Sartorius standard and Low Retention Tips. The test for chemical resistance was performed as described in the Materials and methods using 1000µL pipette tips with Sartorius Picus® electronic pipette (1000µL). The test was repeated for six tips of each manufacturer.

least amount of residue with both volumes. Even Sartorius standard tips retained similar levels of residue as some of the competitors' low retention tips. The use of Sartorius Low Retention Tips resulted in the best pipetting precision, supporting the benefits of these tips (e.g., in PCR setup). The data also shows that the differences in residual liquid amount between the standard and the low retention tips grow with the increase in pipetted volumes. This is due to the liquid adhering to a larger surface area inside the tip.

Effect of Surface Tension

The effect of liquid surface tension on the amount of residue left in the tip, after dispensing, was tested by using various concentrations of isopropanol, which has a low surface tension (23mN/m) (Figure 2). The stronger the Isopropanol concentration and the lower the surface tension, the more beneficial it was to use highly hydrophobic low retention tips rather than standard pipette tips, to minimize the reagent loss and pipetting imprecision. Similar benefits could not be seen with water or other aqueous solutions, due to standard tips already having a clearly lower surface energy (30mN/m) compared to water (72mN/m).

Chemical Resistance of Low Retention Pipette Tips

Several technologies are being used to create low retention surfaces to pipette tips. The most stable methods produce tips, which have complete coverage in terms of hydrophobicity, and are non-leaching. As shown in the Figure 3, there is a significant variance in the tested low retention tips in terms of chemical resistance. With some of the competitor tips the low retention feature was significantly reduced after treatment with the selected solvents. The performance of Sartorius Low Retention Tips after chemical testing was at the same level as without any chemical treatment, suggesting that these tips are inert and chemically resistant. Autoclaving the Sartorius Low Retention Tip also had no effect on the performance of the tips (data not shown).

DISCUSSION

The test results show that Sartorius Low Retention Tips clearly reduce the liquid residue in the tip when handling detergents or other liquids with low surface tension. The data also suggests that the low retention tips available on the market can differ quite significantly in performance and chemical tolerance. Out of the various tips tested, Sartorius Low Retention Tips secured the best sample recoveries, best precision and the best chemical resistance.

Size	Cat. No.	Pack of
0.1–10 µL	**10033-694**	**960**
0.5–200 µL	**10033-698**	**960**
5–350 µL	**10033-686**	**960**
10–1,000 µL	**10033-690**	**960**
50–1,200 µL	**10033-692**	**960**
50–1,200 µL, Ext. Length	**10033-702**	**960**

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All qTOWER³ models include the qTOWER³ system, Analytik Jena's PC-based qPCRsoft software to conduct qPCR and perform analysis, 115V power cable, USB cable, and user manual.

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Description	Cat. No.	Unit
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qTOWER³ touch Real-Time	**75830-236**	**Each**
qTOWER³ G touch Real-Time PCR	**75830-238**	**Each**

This specific product is not available in Canada. Please contact your VWR Sales Representative to learn about easy access to similar options available in your region.

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SeaPlaque® Agarose	125 g	**12001-898**	**Each**
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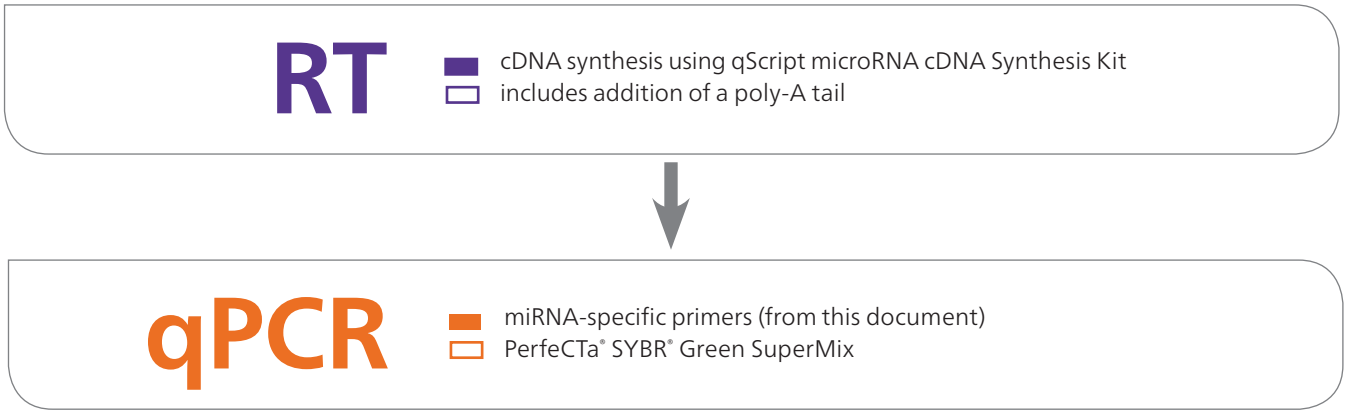
Primer Design Using the qScript® miRNA Quantification System

A key component for quantification of microRNA (miRNA) using real-time quantitative PCR (RT-qPCR) is proper primer design. In order to assist you with using the qScript miRNA quantification system we have prepared this technical guide, which provides details on how to design assay primers specific to your miRNA of interest.

The basic procedure for the qScript microRNA quantification system involves 2-step RT-qPCR and is comprised of three components:



As noted above, two of the components are retail products available through VWR.

The third component, miRNA- specific primers, are the subject of this document. The oligonucleotides resulting from this procedure may be ordered from the vendor of your choice.

Basic Steps for Primer Design

1. Convert miRNA sequence to a DNA sequence
2. Append the reverse complement of the oligo-dT adapter primer (sequence provided below) to its 3' end.
3. Using primer design software or web-based tool, design the miRNA-specific FORWARD PRIMER that is compatible and T_m-balanced with the REVERSE PRIMER (universal primer, sequence provided below).

Example – human miR-193P

1. **miRNA sequence:**
5'-UGUGCAAAUCUAUGCAAAACUGA-3'

Convert to DNA sequence

5'-TGTGCAAATCTATGCAAAACTGA-3'

Quantabio

2. **Oligo dT Adapter Primer:**

5′-GCATAGACCTGAATGGCGGTAAGGGTGTGGTAGGCGAGACATTTTTTTTTTTTTTTTTTT-3′

↓ Make reverse complement

5′-AAAAAAAAAAAAAAAAAAAATGTCTCGCCTACCACACCCTTACCGCCATTCAGGTCTATGC-3′

Template created by appending the RC of adapter primer to miRNA (DNA seq):

5′-TGTGCAAATCTATGCAAAACTGAAAAAAAAAAAAAAAAAAAAATGTCTCGCCTACCACACCCTTACCGCCATTCAGGTCTATGC-3′

3. **Design FORWARD PRIMER** →

ACACGTTTAGATACGTTTGACT

5′-TGTGCAAATCTATGCAAAACTGAAAAAAAAAAAAAAAAAAAAATGTCTCGCCTACCACACCCTTACCGCCATTCAGGTCTATGC-3′

← ATGGCGGTAAGTCCAGATACG

Select last 21 bases as REVERSE PRIMER (Universal Primer)

When designing the forward primer specific to your miRNA, restrict the search to 1–25 bases. In the example above, the software (Oligo7) determined the optimal primer sequence (green) to effectively be the miRNA sequence. In other cases, depending on T_m, balancing and compatibility with the universal primer, it may be longer or shorter.

Key Sequences

Oligo-dT adapter primer

5'-GCATAGACCTGAATGGCGGTAAGGGTGTGGTAGGCGAGACATTTTTTTTTTTTTTTTTTT-3'

Primer design: Used in step 2 for creating the sequence template. Included as part of the qScript microRNA cDNA synthesis kit.

Universal primer

5'-GCATAGACCTGAATGGCGGTA-3'

Primer design: Used in step 3 as the reverse primer. Included with the qScript microRNA cDNA synthesis kit.

Developed Assays

A list of primers for more than 1500 miRNAs have already been designed and are available for download. Please contact your VWR Sales Rep for more information.

Description	Rxn Size	Cat. No.	Unit
PerfeCTa SYBR Supermix	100	**101414-150**	**Each**
PerfeCTa SYBR Supermix	500	**101414-152**	**Each**
PerfeCTa SYBR Supermix	100	**101414-158**	**Each**
PerfeCTa SYBR Supermix	500	**101414-160**	**Each**
PerfeCTa SYBR Supermix	100	**101414-166**	**Each**
PerfeCTa SYBR Supermix	500	**101414-168**	**Each**
qScript MicroRNA cDNA Synthesis Kit	25	**89168-788**	**Each**
qScript MicroRNA cDNA Synthesis Kit	100	**89168-790**	**Each**

DNA Sequencing Made Simple

Widely used worldwide, Sanger sequencing is the Gold Standard in DNA sequencing due to its high accuracy on very long read-lengths using a small amount of target molecules. Next-generation Sequencing (NGS) came, well, next which made it possible to sequence an entire genome in a couple of days. The reads are shorter than in Sanger sequencing, so it can be challenging to align to the reference genome with confidence. Sanger sequencing was used to sequence the Human Genome, but now it is used to validate novel sequences from NGS. However, the process of growing colonies, purifying the DNA prior to the Cycle Sequencing reaction, then purifying again before the Sanger sequencing run is still regarded as time-consuming and tedious.

Starting with a ligation reaction, which doesn't need to be especially high-yielding, the addition of TempliPhi™, which includes Phi29 DNA polymerase, for isothermal Rolling Circle Amplification (RCA) will generate micrograms of high quality DNA. The product of TempliPhi can be used directly in the Cycle Sequencing reaction. Once that reaction is finished, a simple purification step to remove unincorporated dyes and dNTPs via gel filtration, yields a Sanger sequencing-ready template. Specially formulated for Sanger sequencing, AutoSeq™ gel filtration columns, or the high-throughput version called AutoScreen, available in 96-well plate format, can be used for further purification.

There are several NGS platforms with their own unique challenges, but the focus here is on illumina platforms. The easiest part of NGS is hitting the "Sequence" button on your HiSeq. The challenge lies in the steps that lead up to hitting that button.

To obtain the greatest coverage across the whole genome, or even just the whole exome, start with as much full length genomic DNA as possible. As part of the library prep process, the gDNA is fragmented, end-repaired, and A-tailed before the DNA adaptors are ligated to each end. Several rounds of PCR using the adaptor sequences as forward and reverse priming sites are performed, followed by magnetic bead clean-up and elution for attachment to the flow-cell. A large amount of these sequence-ready pieces of DNA must be generated to attain full coverage, but with each step of the library prep process, there is risk of DNA template loss, resulting in coverage gaps. Those gaps could include an important single or multi-nucleotide variant, insertion/deletion ("Indel"), or transposed element that may hold the key to a disease pathway. The goal is to limit the number of gaps. To achieve this, the depth can be increased — the amount of times that the material is sequenced, e.g. 30X, 100X, etc. — but the results are still limited to the material attached to the flow-cell. In addition, it's very expensive to perform even 30X, let alone 100X. However, there is a better option.

Phi29 Whole Genome Amplification

Phi29 is capable of performing whole genome amplification on linear DNA as well as circular DNA. This is called multiple-strand displacement amplification or MDA. Full length human genomic DNA can be amplified by GenomiPhi™, GE Healthcare's MDA kit, yielding micrograms of DNA from nanograms. This process enables numerous downstream analyses from a single sample: PCR, qPCR, microarray, and sequencing. Making lots of DNA decreases the chance of having gaps in NGS coverage. Using GenomiPhi prior to NGS library prep is standard in any sequencing lab. GenomiPhi can also be used prior to Sanger sequencing, too. TempliPhi amplifies a circle of DNA very

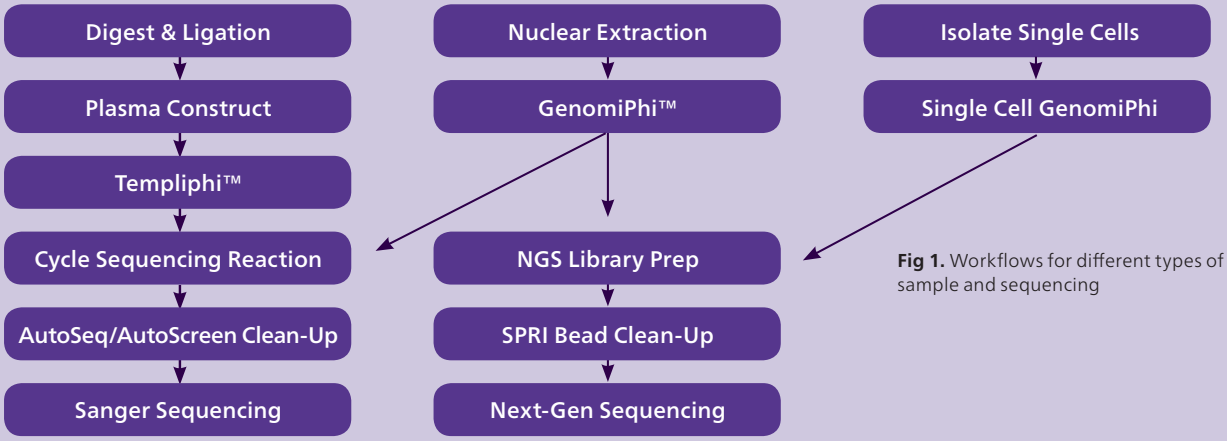


Fig 1. Workflows for different types of sample and sequencing

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efficiently because the primers that bind and guide the reaction, just sweep around and around the circle, spinning out long stretches of DNA. The GenomiPhi amplification of linear DNA is less efficient and requires multiple binding sites across the genome. One way to ensure that the DNA will be amplified is for the template to be greater than 1Kb.

Better Extraction Methods

Even though phenol is dangerous to use and even prohibited at many research sites, researchers are returning to phenol: chloroform, because the quality and length of template that can be obtained is unmatched, and researchers want to avoid the risk of loss, fragmentation, or damage to the template.

The specific material that DNA is being extracted from pose additional challenges. For plants and seeds, the challenge is to remove all polysaccharides without damaging the template. For cancer researchers, the challenge is to extract DNA from Formalin-Fixed Paraffin-Embedded (FFPE) tumor samples in order to trace possible tumorigenic mutations to archived tumors.

What researchers need is a kit that can give them the quality and length of a template created with Phenol:Chloroform, but without the phenol. GE Healthcare's Nucleon kits offer the ease-of-use, quality and length of template provided by the phenol:chloroform process, but do not use phenol. The extracted DNA from the nucleon kit when used in combination with GenomiPhi gives the added assurance the researcher needs to reduce potential sequencing coverage gaps.

Single Cells or Trace Amounts of DNA Template

Up to this point, our knowledge of the genome has been limited to populations of cells. However, genetic variation between individual cells was impossible to characterize. You may ask, why is this important? One important example is the formation of a tumor. Somatic mutations randomly occur when cells undergo mitosis. However, most somatic mutations occur in a part of the genome that will have no downstream effect on gene expression. A tumor generally starts from the accumulation of somatic mutations. To understand understand when and where a tumor will form, the heterogeneity of a tumor must be characterized. With our ability to isolate single cells, lyse them open to release their genome, and sequence it by NGS, we come closer to the possibility of predicting where and when a tumor will form. The challenge here is the amount of available DNA. A single human cell only has about 7pg of DNA. Single Cell GenomiPhi kit lyses the cell and amplifies its entire genome in a single tube in about two hours. The typical yield is from 4–7µg, which is more than enough to sequence the entire genome from a single cell. The gDNA of five cells was amplified with Single Cell GenomiPhi, which gave >90% on-target exome sequence coverage when run in whole exome sequencing (10X coverage). This result is equivalent in performance of unamplified gDNA control (>98%).

Summary

Whatever type of DNA that needs to be sequenced, with either method, GE Healthcare Life Sciences offers innovative products to enable high quality results, quickly and easily.

DNA Sequencing Made Simple

GE Healthcare Life Sciences Offers Innovative Products to Enable High Quality Results, Quickly and Easily

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- Prepare circular DNA templates for cycle sequencing, cloning and transformation in 4–6 hours
- Use amphlified DNA directly for cycle sequencing without purification
- Generatate micrograms quantities of template DNA from picogram amounts of starting material

TempliPhi™ Kits use a unique process to efficiently prepare micrograms of circular DNA from picogram input material. The DNA templates are prepared by Rolling Circle Amplification (RCA) using bacteriophage Phi29 DNA polymerase. TempliPhi uses an isothermal method for the exponential amplification of circular DNA. Phi29 DNA polymerase is active at 30°C, enabling amplification to be performed at this temperature without the need for thermal cycling. The TempliPhi protocol requires less than 20 min of hands-on time to amplify 96 samples from bacterial colonies.

Description	Size	Cat No.	Unit
TempliPhi 100	100 rxn	**89131-608**	**Each**
TempliPhi 2000	100 rxn	**89134-006**	**Each**
GenomiPhi™ V2	100 rxn	**95040-352**	**Each**
Single Cell GenomiPhi	100 rxn	**10146-198**	**Each**
Nucleon Blood & Cultured Cells	50 rxn	**95017-486**	**Each**

Optimization of DNA, RNA, and RNP Delivery for Efficient Mammalian Cell Engineering Using CRISPR/Cas9

Chuenchanok Khodthong, Josh Snow, and Laura Juckem*
Mirus Bio LLC, Madison, Wisconsin USA

ABSTRACT

The CRISPR/Cas9 genome-editing platform is a versatile and powerful technology to efficiently create genetically engineered living cells and organisms. This system requires a complex of Cas9 endonuclease protein with a gene-targeting guide RNA (gRNA) to introduce double-strand DNA breaks (DSBs) at specific locations in the genome. The cell then repairs the resulting DSBs using either homology-directed repair (HDR) or the error-prone non-homologous end joining (NHEJ) pathway. Both DNA repair pathways can be leveraged in different ways to introduce desired modifications at the target locus.

The success of CRISPR genome editing experiments is limited by the intracellular delivery and expression of Cas9 protein and gRNA. Many methods for achieving Cas9-mediated cleavage have been identified, and the choice of DNA, RNA, or ribonucleoprotein (RNP) format is dictated by experimental goal and cell type. Transfection of each type of molecule requires specific considerations for efficient functional delivery. We performed transfections using different combinations of molecules including: plasmid DNA, messenger RNA, Cas9 protein, and gRNA to maximize targeting of the Cyclophilin B (PPIB) gene in HEK 293T/17, U2OS, and other mammalian cell types. Our results extend the utility of the CRISPR/Cas9 system by identifying optimal transfection conditions for intracellular delivery of Cas9 and gRNA in different formats.

INTRODUCTION

Genome editing technologies such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated protein 9) system have proven to be invaluable research tools for generating precise genome alterations. All three systems enable DNA modifications by creating a double-stranded break (DSB) at the target locus. ZFNs and TALENs both utilize endonuclease domains that are fused to modular DNA binding proteins and can be programmed to target specific genomic locations. In contrast to ZFNs and TALENs, which rely on protein-DNA interactions to afford target recognition, Cas9 is targeted to DNA via a short guide RNA that forms Watson-Crick base pairs with the target DNA[1-3]. Targeting of Cas9 to different loci only requires delivery of Cas9 and the appropriate guide RNA sequence, making the CRISPR/Cas9 system a highly facile technology for genome engineering[4,5].

The development of CRISPR/Cas9 technology into a genome editing platform stems from basic research into the mechanisms

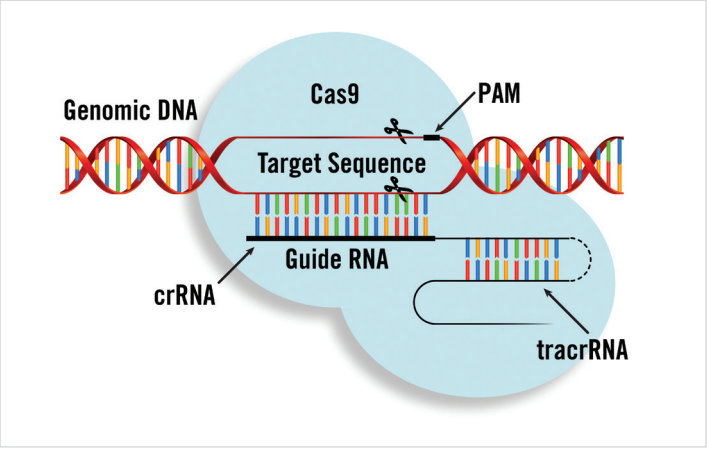

Figure 1. CRISPR/Cas9 Genome Editing. The Cas9 endonuclease (blue) is targeted to DNA by a guide RNA which can be supplied as a two-part system consisting of crRNA and tracrRNA or as a single guide RNA, where the crRNA and tracrRNA are connected by a linker (dotted line). Target recognition is facilitated by the protospacer-adjacent motif (PAM). Cleavage occurs on both strands (scissors) 3bp upstream of the PAM.

of adaptive immunity in prokaryotes[6]. In bacteria and archaea, CRISPR arrays and Cas genes normally function to provide protection against viral and plasmid invasion by targeting and silencing foreign nucleic acids in a sequence-specific manner[6,7]. CRISPR arrays are genetic segments characterized by identical repeats interspersed with non-repetitive sequences called spacers. The sequence of each spacer corresponds to fragments of foreign DNA (protospacers) that is acquired through exposure to previous viral or plasmid DNA. Upon subsequent viral and plasmid invasion, the CRISPR locus is transcribed and processed into short CRISPR RNAs (crRNAs) that guide the Cas endonuclease to digest the complementary genomic target sequence of the invading pathogens. CRISPR–Cas systems are highly diverse and can be classified into three major types (I–III). In type I and II systems, a short nucleotide sequence adjacent to the protospacer, called the protospacer adjacent motif (PAM), is required for target recognition. The PAM is crucial for the CRISPR–Cas system to discriminate between the invading pathogen genome and the CRISPR locus in the host genome, which does not contain the PAM[8].

The CRISPR type II system from *Streptococcus pyogenes* was the first system to be adapted for genome engineering in mammalian cells by inducing sequence-specific DSBs at desired genomic locations[3]. In this system, the guide RNA consists of two parts: the CRISPR RNA (crRNA), which forms base pairs with the

Delivery by Mirus

target DNA, and a trans-activating crRNA (tracrRNA), which forms base pairs with the crRNA and triggers Cas9 cleavage of target DNA. In order to perform genome editing, the Cas9 endonuclease and the two-part gRNA, consisting of a constant tracrRNA and a target specific crRNA, must be introduced to cells. The guide RNA can be supplied separately as crRNA and tracrRNA or as a single chimeric oligonucleotide referred to as a single guide RNA (sgRNA). Once inside the cell, the protospacer sequence at the 5′ end of the crRNA will direct Cas9 to a specific target DNA site immediately 5′ of a PAM and guide double-strand DNA cleavage (Figure 1). Cas9-induced DSBs are repaired by either homology-directed repair (HDR) or nonhomologous end joining (NHEJ). In HDR, DNA lesions are repaired using homologous sequence as a template. Therefore, researchers can take advantage of this repair pathway to make specific alterations in the host genome by supplying a donor DNA template that has homology with the sequence flanking the DSB. The NHEJ repair mechanism is more error prone and often results in the introduction of small insertions and deletions (indels) causing frameshift mutations or premature stop codons to produce gene knockouts[6].

The appropriate delivery method of Cas9 and gRNA is critical for performing effective genome editing in the targeted cells. Current systems for delivering Cas9 include plasmid DNA, mRNA, and ribonucleoprotein (RNP) complex. Each method has its own set of advantages and limitations. The delivery of Cas9 as plasmid DNA offers a simple and low-cost approach; however, many studies have shown a higher degree of off-target cleavage events using this method[10,11]. Transfection of Cas9 mRNA enables rapid gene expression and eliminates the risk of insertional mutagenesis[12]. Delivery of Cas9 protein/guide RNA ribonucleoprotein (RNP) complex results in the most rapid pulse of genome editing activity and reduces the possibility of off-target cleavage events[13]. Cas9 mRNA and RNP formats provide an efficient delivery method to cell types that are resistant to transfection with plasmid DNA[7]. To date, the delivery of the Cas9 and gRNA remains challenging in many cell types, particularly primary and suspension cells[7]. To overcome these limitations, we optimized conditions for transfection of CRISPR/Cas9 components using combinations of plasmid DNA, mRNA, Cas9 protein, and gRNA in a variety of mammalian cells (Figure 2). Our

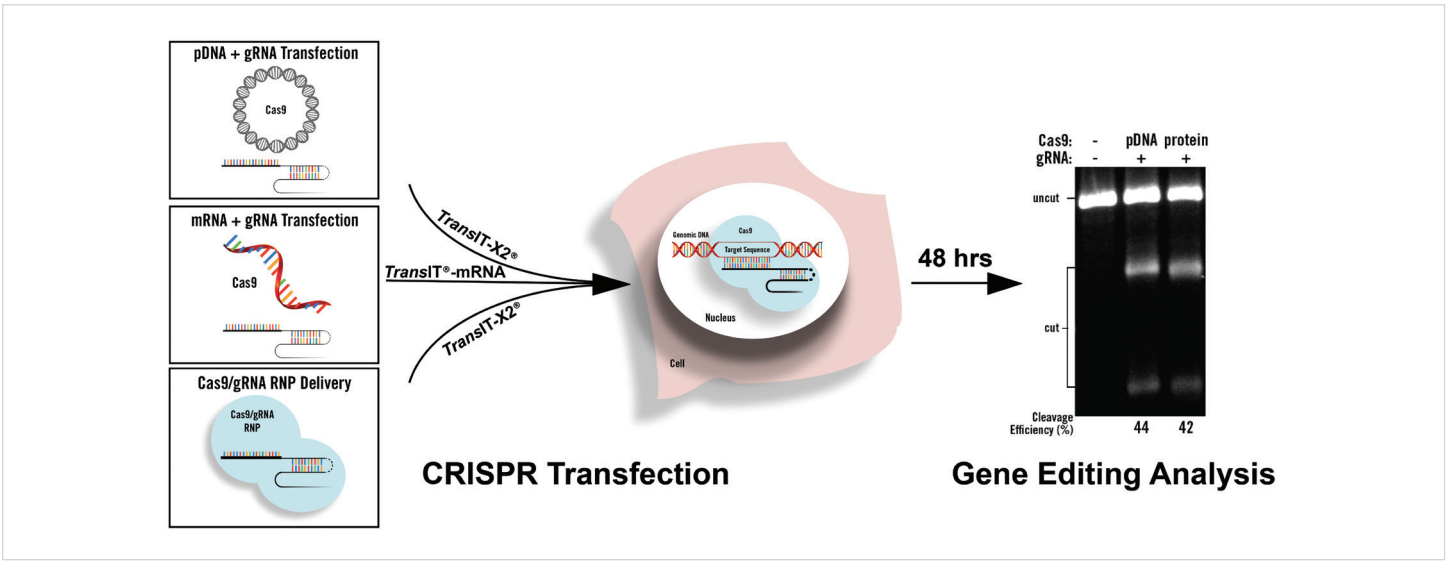

Figure 2. Cas9 Encoding Plasmid, mRNA, or Protein and Synthetic crRNA:tracrRNA Co-transfection Optimization Experimental Workflow. gRNA targeting PP/8 gene was formed by incubating synthetic tracrRNA (Dharmacon) and synthetic crRNAs targeting PPIB gene in exon 2 (Dharmacon) in Opti-MEM® serum-free medium (ThermoFisher Scientific) for 10 minutes at room temperature. The PPIB targeting gRNA was then co-transfected into several different mammalian cell lines with either Cas9 encoding plasmid (pCas9-GFP; MilliporeSigma) or Cas9 encoding mRNA (Cas9 mRNA with 5-methylcytidine and pseudouridine modification; Trilink BioTechnologies) using *Trans*IT-X2 Dynamic Delivery System (Mirus Bio) or *Trans*IT-mRNA Transfection Kit (Mirus Bio) respectively. Alternatively, PPIB targeting gRNA was used to form an RNP complex with purified bacterial expressed recombinant Cas9 protein (PNA Bio) by incubating the two components in Opti-MEM serum-free media for 10 minutes at room temperature. The PPIB targeting Cas9 RNP was then transfected into several different mammalian cell lines using the *Trans*IT-X2 Dynamic Delivery System. Transfection optimization was performed in a 24-well plate format varying the transfection reagent amount, the concentration of gRNA, and Cas9 encoding molecule. Genomic DNA was then harvested from the transfected cells at 48 hours post-transfection and the region spanning the gRNA target site was PCR amplified and analyzed for level of gene editing efficiency by a mismatch detection assay using T7 Endonuclease I (New England Biolabs).

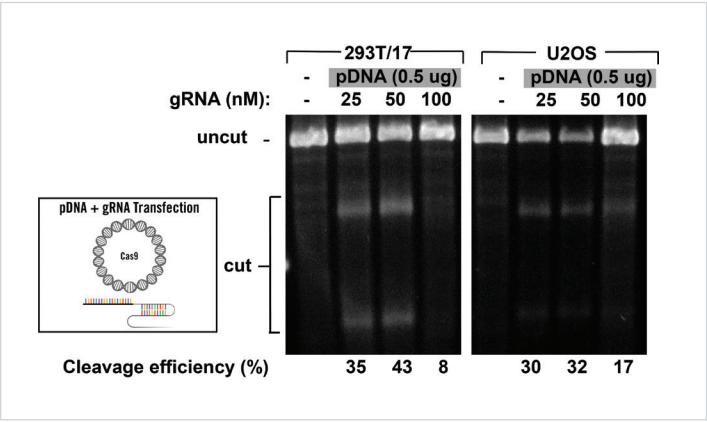

Figure 3. Efficient Genome Editing with Cas9 Plasmid DNA and gRNA Oligonucleotides. HEK293T/17 and U20S cells were co-transfected with 0.5µg of Cas9 encoding pDNA and 50nM PPIB targeting 2-part gRNA using *Trans*IT-X2 Dynamic Delivery System (2µL/well of a 24-well plate). A T7EI mismatch detection assay was used to measure cleavage efficiency at 48 hours post-transfection.

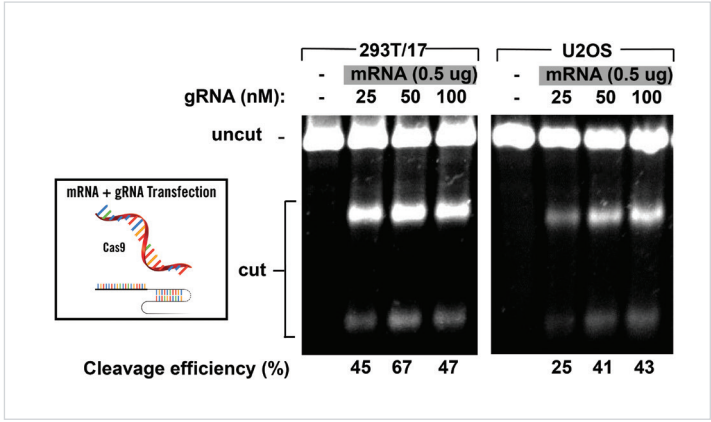

Figure 4. Efficient Genome Editing with Cas9 mRNA and gRNA Oligonucleotides. HEK293T/17 and U20S cells were co-transfected with 0.5µg of Cas9 encoding mRNA, Smee, Ψ, and 25nM of PPIB targeting 2-part gRNA using *Trans*IT-mRNA Transfection Kit (0.5µL/well of 24-well plate of both mRNA Reagent and Boost Reagent). A T7EI mismatch detection assay was used to measure cleavage efficiency at 48 hours post-transfection.

results identify the most effective methods for intracellular delivery of Cas9 and gRNA in hard-to-transfect cells.

RESULTS

Transfection Optimization for Cas9 Mediated Genome Editing by Plasmid and gRNA Delivery

Transfection optimization was performed in HEK293T/17 and U2OS in a 24-well plate format using the *Trans*IT-X2® Dynamic Delivery System. Cells were co-transfected with 0.5µg of Cas9 encoding plasmid and varying concentrations of gRNA targeting the human *PPIB* gene. 2µL of *Trans*IT-X2® Dynamic Delivery System was used per well of a 24-well plate. Genomic DNA was harvested from the transfected cells at 48 hours post-transfection. The region spanning the gRNA target site was PCR amplified and analyzed for gene editing efficiency by a mismatch detection assay using T7 Endonuclease I (T7EI). Under these conditions, the concentration of gRNA complex that yielded the highest gene editing efficiency in HEK293T/17 and U2OS cells is between 25-50nM yielding cleavage efficiencies of 43 and 32%, respectively (Figure 3).

Transfection Optimization for Cas9 Mediated Genome Editing by mRNA and gRNA Delivery

Cas9 mRNA and two-part gRNA transfection optimization was performed in HEK293T/17 and U2OS in a 24-well plate format using *Trans*IT®-mRNA Transfection Kit. Cells were co-transfected with 0.5µg of Cas9 encoding mRNA (5-methyl cytidine, pseudouridine) and varying concentrations of gRNA targeting the human PPIB gene. 0.5µL of *Trans*IT®-mRNA Transfection Reagent and 0.5µL of mRNA Boost Reagent was used per well of a 24-well plate. The use of modified base mRNA decreases the innate immune response to long RNA and is also believed to enhance translation, thereby increasing overall expression levels[17]. Genomic DNA was then harvested from the transfected cells at 48 hours post-transfection. The region spanning the gRNA target site was PCR amplified and analyzed for level of gene editing efficiency by a mismatch detection assay using T7EI. The concentration of gRNA complex that yields the highest gene editing efficiency in

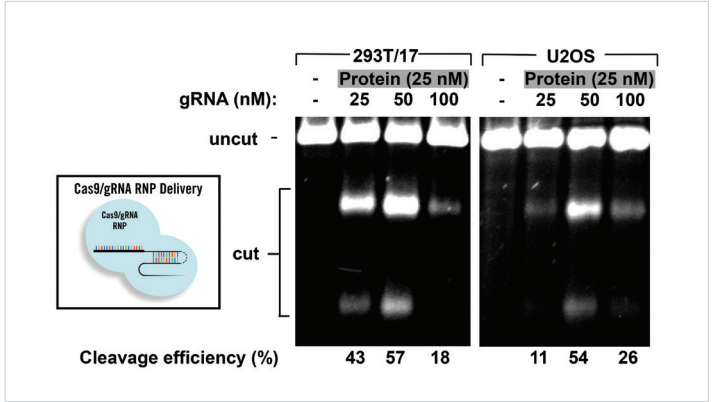

Figure 5. Efficient Genome Editing with Cas9 + gRNA Ribonucleoprotein Complexes. The RNP complex of PPIB targeting 2-part gRNA and Cas9 protein was delivered into HEK293T/17 and U20S cells using *Trans*IT-X2 Dynamic Delivery System (1µL/well of a 24-well plate). A T7EI mismatch detection assay was used to measure cleavage efficiency at 48 hours post transfection. High levels of gene editing are achieved in cells that were transfected with an RNP complex comprised of 50nM of gRNA and 25nM of Cas9 protein.

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HEK293T/17 and U2OS cells is 50nM, yielding cleavage efficiencies of 67 and 41%, respectively (Figure 4).

Transfection Optimization for Cas9 Mediated Genome Editing by Cas9 RNP Complex Delivery

RNP complex transfection optimization was performed in HEK293T/17 and U2OS in a 24-well plate format using *Trans*IT-X2 Dynamic Delivery System. To form an effective RNP complex, first, two part gRNA (crRNA and tracrRNA) were incubated in Opti-MEM® serum-free medium for 10 minutes at room temperature; second, Cas9 protein was added and incubated for 10 minutes; third, *Trans*IT-X2 Dynamic Delivery System was added and incubated for 15 minutes. Finally, the transfection complex was added to cells growing in normal growth medium. Genomic DNA was harvested from the transfected cells at 48 hours post-transfection. The region spanning the gRNA target site was PCR amplified and analyzed with a mismatch detection assay using T7EI to determine the level of gene editing efficiency. The optimal level of gene editing is achieved in HEK293T/17 and U2OS by delivery of a Cas9 RNP complex comprised of 25nM Cas9 protein and 50nM gRNA, resulting in cleavage efficiency of 57 and 54%, respectively (Figure 5).

Cas9 Mediated Genome Editing in Hard-to-Transfect Cells

Empirical evidence has revealed that some cell types are resistant to chemical transfection methods. There is a lack of understanding as to why these cells are hard to transfect, but it may be dependent on the type of macromolecule that is being delivered. Cell types that frequently fall into this category include: primary cells, hematopoietic, and stem cells. To address the need for genome editing in these areas, we used three cell types, K562, primary Normal Human Dermal Fibroblasts (NHDF), and induced human pluripotent stem cells (HiPSC) as models for hard-to-transfect cell types. Transfection optimization was performed in K562, primary NHDF, and Human iPSC cells. K562 and primary NHDF cells growing in a 24-well plate were co-transfected with either 0.5µg of Cas9 encoding plasmid, 0.5µg of Cas9 encoding mRNA, or 25nM of Cas9 protein, plus varying concentrations of PPIB targeting gRNA delivered using the *Trans*IT-X2 Dynamic Delivery System (plasmid DNA and RNP) or *Trans*IT-mRNA Transfection Kit (mRNA). Human iPSC (ATCC-DYS100) were grown in a 6-well plate and co-transfected with varying amounts of Cas9 encoding plasmid, 2µg of Cas9 encoding mRNA, varying concentrations of Cas9 protein, and PPIB targeting gRNA with varying amounts of *Trans*IT-X2 Dynamic Delivery System or *Trans*IT-mRNA Transfection Kit. After 48 hours, the relative level of gene editing efficiency was

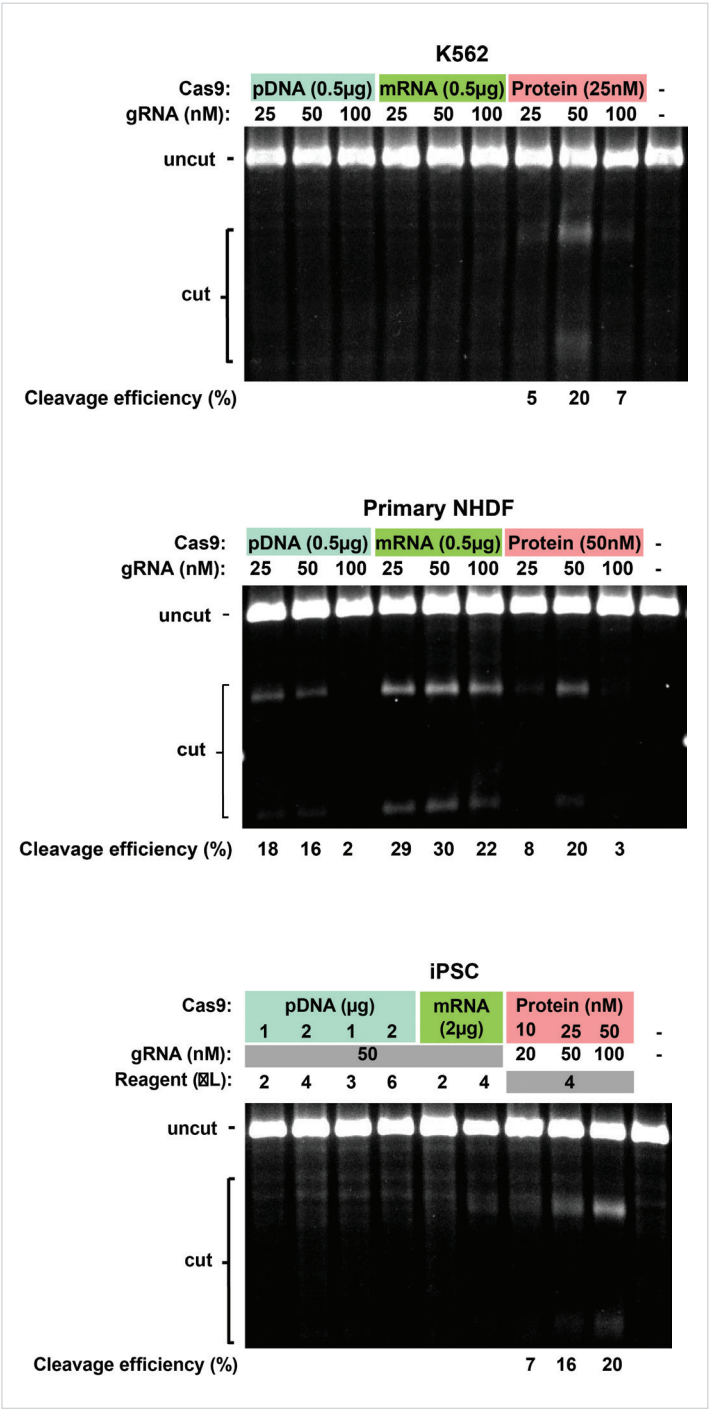

Figure 6. Efficient Genome Editing in Hard to Transfect Cells. *Trans*IT-X2 Dynamic Delivery System was used to deliver Cas9 pDNA/gRNA and Cas9 protein gRNA (RNP complex). *Trans*IT-mRNA Transfection Kit was used to deliver Cas9 mRNA/gRNA into KS62, primary NHDF, and Human iPSC cells. A T7EI mismatch assay was used to measure cleavage efficiency at 48 hours post-transfection.

assessed by a mismatch detection assay using T7EI. The highest level of gene editing in K562 and iPSC cells is achieved by delivery of a Cas9 RNP complex composed of 25nM Cas9 protein and 50nM gRNA, yielding cleavage efficiencies up to 20%. In contrast, the optimum level of gene editing is attained in primary NHDF cells co-transfected with 0.5 mg of Cas9 encoding mRNA and 25-50nM gRNA to yield 30% cleavage efficiency (Figure 6).

CONCLUSIONS

The ability to utilize targeted nucleases to generate precise modifications in the genome holds great promise for basic research as well as gene and cellular therapies. Emerging therapeutic strategies using the CRISPR/Cas9 system to modify nucleic acids within disease-affected cells could enable the treatment of debilitating genetic diseases such as cystic fibrosis[14] and Duchenne muscular dystrophy[15]. Furthermore, it could also be utilized to generate mutations that protect cells against infection. For example, HIV infection could be blocked through a loss of function mutation generated in the CCR5 receptor[16]. Efficient methods for delivery of Cas9 and guide RNA are essential to realizing the full potential of the CRISPR genome editing platform. Here we have optimized Cas9 and gRNA delivery methods using the *Trans*IT-X2 Dynamic Delivery System and the *Trans*IT-mRNA Transfection Kit for generating desired gene alterations in a variety of mammalian cells including some difficult to transfect cells such as HiPSCs, K562, and primary NHDF. Several experimental parameters influencing the efficiency of genome editing were optimized to achieve the highest level of genome editing in each particular cell type. Numerous laboratories have demonstrated that the CRISPR/Cas9 system is robust and efficient. Researchers have flexibility in their experimental design which includes the delivery of Cas9 endonuclease in a variety of formats including: plasmid DNA, mRNA, and protein via a RNP complex. There are advantages and disadvantages to each system such as: cleavage efficiency, cost, and specificity. Herein, we have demonstrated that RNP formats for Cas9 delivery offers the highest gene editing efficiency, particularly in hard-to-transfect cells.

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Size	Cat. No.
TransIT-X2® Dynamic Delivery System	
0.3 mL	**10766-890**
0.75 mL	**10766-892**
1.5 mL	**10766-888**
TransIT®-mRNA Transfection Kit	
0.4 mL	**10767-106**
1.0 mL	**10767-108**
Ingenio® Electroporation Solution	
25 Rxn	**10766-836**
50 Rxn	**10766-842**
100 Rxn	**10766-848**

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Superior Cloning Performance with SGI-DNA Gibson Assembly® Kits

SGI-DNA
A Synthetic Genomics, Inc. Company

Christine Chen, Ph.D., Synthetic Genomics, Inc., La Jolla, CA

Introduction

Molecular cloning techniques have evolved rapidly over the last decade, particularly with the development, adoption, and refinement of seamless cloning strategies that allow for the scarless insertion of DNA fragments into a vector. In addition to leaving the insert sequences fully intact, seamless cloning technologies offer the added advantage of being faster than traditional restriction enzyme digest-based cloning. One seamless cloning strategy in particular, Gibson Assembly® seamless cloning, has been extensively embraced by the life science community, as evidenced by over 1200 citations of the manuscript[1] originally describing the technique.

Because of its ease-of-use and efficiency, the Gibson Assembly method is ideally suited for routine cloning. In addition to the straightforward cloning of a single insert with a single vector, the Gibson Assembly method is the ideal choice for complex assembly projects, such as the simultaneous assembly of multiple inserts with a vector, as depicted in Figure 1. The Gibson Assembly method offers substantial time savings for multiple-insert assembly projects, which would typically require multiple rounds of traditional restriction enzyme digest-based cloning.

Gibson Assembly was first developed by Daniel Gibson and colleagues at the J. Craig Venter Institute. In the ensuing years, Daniel Gibson and his team have been refining and improving the technique and reagent formulations at Synthetic Genomics, Inc. (SGI). The results of their expertise and years of development are available commercially as the SGI-DNA Gibson Assembly HiFi 1-Step and Ultra kits. Although several other seamless cloning kits are commercially available, only the SGI-DNA Gibson Assembly kits use the precise reagent formulation invented and further refined by Dr. Daniel Gibson.

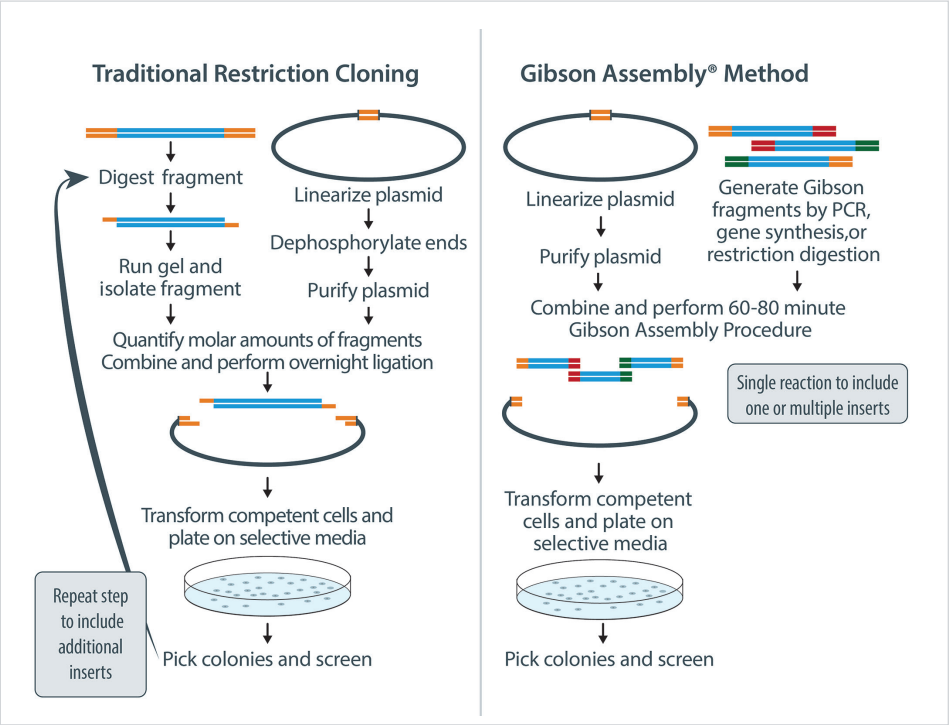

Figure 1. The Gibson Assembly Method is faster and more efficient than traditional cloning. Traditional restriction cloning using compatible restriction endonucleases requires 1–2 days of preparative steps to generate cloning ends on the insert and plasmid. Typically, only one insert can be ligated into the plasmid at a time. Generating longer inserts usually requires multiple rounds of restriction and ligation. The Gibson Assembly Method allows for several inserts to be simultaneously assembled in a single reaction that takes only 1 hour or less, allowing for the rapid generation of very large constructs. The Gibson Assembly Method requires a linearized vector and 20–80bp sequence overlaps at the ends of the DNA elements to be assembled. Overlap sequences are intrinsic to the construct(s) and plasmid, eliminating the need for specific restriction sites.

Evaluating Commercially Available Seamless Assembly Kits

To evaluate the performance of SGI-DNA Gibson Assembly HiFi 1-Step and Ultra kits in multi-fragment assembly reactions, five 800bp fragments were assembled into an 8kb vector using the HiFi or Ultra kit and three other commercially available seam-less cloning kits. Assembly reactions were performed according to each manufacturer's protocol, including the relative amounts of insert and vector DNA used for the assembly reaction. Data are presented as the mean of two experiments performed in triplicate. Results are presented in Figures 2 (HiFi 1-Step Kit) and 3 (Ultra Kit).

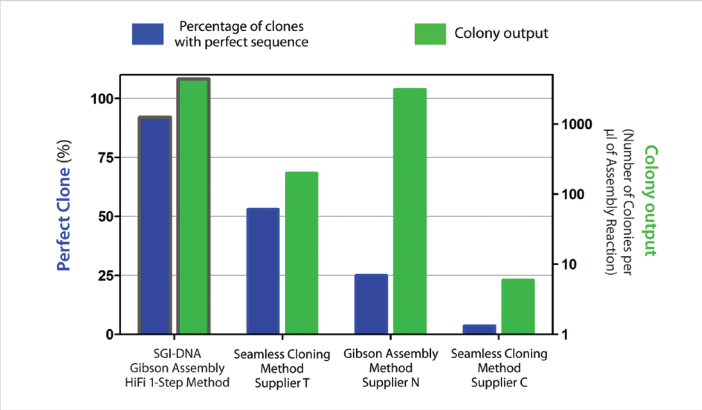

Figure 2. SGI-DNA Gibson Assembly HiFi 1-Step kit exhibits superior results. Five 800bp fragments were assembled into an 8kb vector using the Gibson Assembly HiFi 1-Step kit and three other commercial kits, here called Supplier T, N, or C. The assembly protocol (including relative amounts of DNA used in the assembly reaction) supplied by the manufacturer was followed for each respective kit. Following assembly and transformation, the number of colonies was counted and normalized to the volume of the assembly reaction used for transformation. Twelve colonies were randomly selected for colony PCR. Only positive colonies were utilized for sequencing. The percentage of clones containing perfect sequence is shown in the figure above. Data are presented as the mean value from two assembly experiments performed in triplicate.

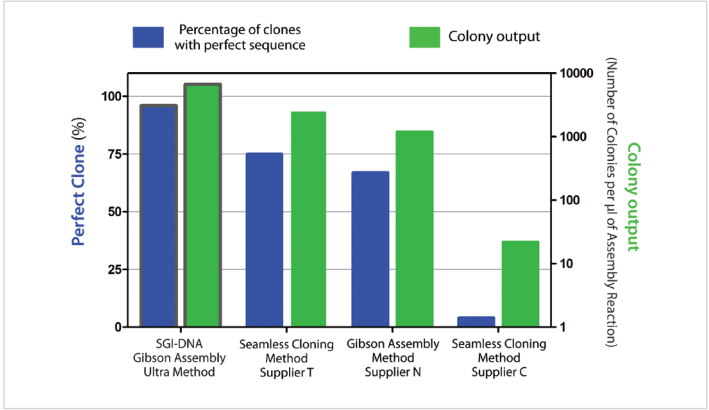

Figure 3. SGI-DNA Gibson Assembly Ultra kit exhibits superior results. Five 800bp fragments were assembled into an 8kb vector using the Ultra kit and three other commercial kits, here called Supplier T, N, or C. The assembly protocol (including relative amounts of DNA used in the assembly reaction) supplied by the manufacturer was followed for each respective kit. Following assembly and transformation, the number of colonies was counted and normalized to the volume of the assembly reaction used for transformation. Twelve colonies were randomly selected for colony PCR. Only positive colonies were utilized for sequencing. The percentage of clones containing perfect sequence is shown in the figure above. Data are presented as the mean value from two assembly experiments performed in triplicate.

Results and Summary

Compared to the other commercially available seamless cloning kits used for these 5-fragment assembly experiments, the SGI-DNA Gibson Assembly HiFi 1-Step and Ultra kits exhibit the highest colony output and sequence accuracy. As shown in Figure 2, the Gibson Assembly HiFi 1-Step Kit delivers the highest percentage of perfect clones — on average, 92% of analyzed clones exhibited perfect sequence. The Gibson Assembly Ultra Kit also delivers the highest percentage of perfect clones (96%) in comparison to the other kits used for multi-fragment assembly reactions (Figure 3). Additionally, like the HiFi 1-Step kit, the Ultra kit exhibits superior colony output. The HiFi 1-Step and Ultra kits also demonstrate similar robust performance when used for single insert cloning, exhibiting the highest cloning efficiency of all kits tested (data not shown).

When selecting a cloning strategy, many options are available. To achieve fast, accurate, and efficient results, SGI-DNA Gibson Assembly HiFi 1-Step and Ultra kits are optimal. The HiFi 1-Step Kit achieves fast assembly (1 hour reaction at a single temperature) and is recommended for assemblies with ≤5 fragments. The Ultra kit is recommended for more complex assemblies of up to 15 fragments, achieving assembly in 1 hour and 20 minutes. The Ultra Kit is also compatible with a broad range of DNA fragment sizes (100bp–100kb). Key features of the kits are shown in Table 1. The crucial advantage of both the HiFi 1-Step and Ultra kits is the ability to clone genes of interest seamlessly, quickly, and accurately.

Reference

1. Gibson, D.G. et al. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods 6: 343–345.

Feature	Gibson Assembly® HiFi 1-Step Kit	Gibson Assembly® Ultra Kit
Hands on Time	<5 minutes	<6 minutes
Reaction Time	60 minutes	80 minutes
Number of Steps	1	2
Fragment Size Range	500bp–32kb	100bp–100kb
Cloning Efficiency	>90%	~96%
Fragments Per Reaction	up to 5	up to 15
Maximum Construct Size	100kb (multi-stage reactions)	1Mb (multi-stage reactions)
Key Advantages	• Quick and easy • Clone up to 5 fragments in a single reaction • Proofreading polymerase reduces chance of mutations at cloning junctions	• Robust and efficient • Clone up to 15 fragments in a single reaction • Suitable for small and large fragments

Table 1. Key Features of SGI-DNA Gibson Assembly Kits.

Gibson Assembly™ Cloning: Single Round Cloning of Multiple DNA Fragments into Any Vector

SGIDNA
A Synthetic Genomics, Inc. Company

Best Performing Seamless Cloning Kits and Master Mixes From The Inventors of Gibson Assembly

- Choose the reliable Gibson Assembly HiFi 1-Step method or robust Gibson Assembly Ultra Two-Step method
- Ideal for simple or complex constructs — clone 1 to 15 fragments from 100 bp to 100 kb in a single round
- Superior performance — achieve cloning efficienies of up to 95%

Gibson Assembly simplifies the cloning of double stranded DNA fragments into plasmid or BAC vectors. Unlike traditional methods, Gibson Assembly allows any set of fragments to be joined without the need for compatible restriction sites. In contrast to methods requiring specialized cloning vectors, Gibson Assembly allows you to clone in to the vector of your choice. Use Gibson Assembly mixes refined and optimized by Dr. Dan Gibson to avoid subcloning and accelerate cloning and mutagenesis workflows.

Description	Reaction Size	Cat. No.	Unit
Gibson Assembly® HiFi 1-Step Kit	10 rxns	10820-610	10
Gibson Assembly® HiFi 1-Step Kit	50 rxns	10820-614	50
Gibson Assembly® HiFi 1-Step Master Mix	10 rxns	10820-612	10
Gibson Assembly® HiFi 1-Step Master Mix	50 rxns	10820-616	50
Gibson Assembly® Ultra Kit	10 rxns	10820-790	10
Gibson Assembly® Ultra Kit	50 rxns	10820-794	50
Gibson Assembly® Ultra Master Mix	10 rxns	10820-792	10
Gibson Assembly® Ultra Master Mix	50 rxns	10820-652	50

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12 reactions, SOLOs	89005-078	Each
24 reactions, SOLOs	89005-080	Each
48 reactions, SOLOs	89005-082	Each
12 reactions, DUOs	89002-674	Each
24 reactions, DUOs	89002-676	Each
48 reactions, DUOs	89002-678	Each
96 reactions, DUOs	89002-680	Each
TransforMax™ EPI300™ Electrocompetent E. coli (≥1 x 10^{10} cfu/µg DNA)		
20 reactions, 10 x 100 µL	75927-928	Each
50 reactions, 50 x 100 µL	75927-930	Each

NutriStem® hPSC XF Medium Supports Long-Term Culture of Human Pluripotent Stem Cells as Clumps on Corning® Matrigel® Matrix and Single Cells on Corning PureCoat™ rLaminin-521 Cultureware

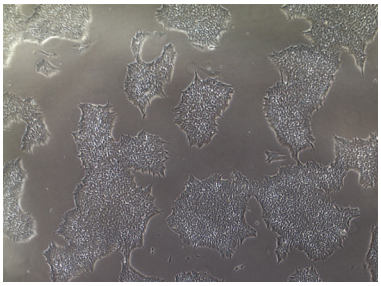
Jeff Partridge, Himabindu Nandivada, Paula Flaherty, and Deepa Saxena Corning Incorporated, Life Sciences, Bedford, MA USA

INTRODUCTION

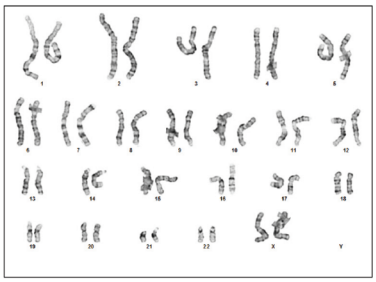
Human pluripotent stem cells (hPSCs) including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), are typically cultured using complex media containing animal-derived components. In addition, adherent culture of hPSCs requires either a layer of feeder cells (e.g., mouse or human fibroblasts), a complex mixture of naturally derived extracellular matrices (e.g., Corning® Matrigel® matrix) or defined surfaces (e.g., recombinant Laminin). In this study, we demonstrated long-term culture of hiPSCs in NutriStem® hPSC XF medium — a serum-free, xeno-free medium — widely used for long-term growth and expansion of hPSCs. Human iPSCs were cultured as clumps on Matrigel matrix or as single cells on Corning PureCoat™ rLaminin-521 cultureware in NutriStem medium for at least ten passages.

Cells exhibited typical hPSC morphology and remained undifferentiated as demonstrated by the expression of Oct-3/4 (>94%), SSEA-4 (>95%) and the absence of SSEA-1. After ten passages, pluripotency was shown by differentiation into the three germ layers and cells retained a normal karyotype.

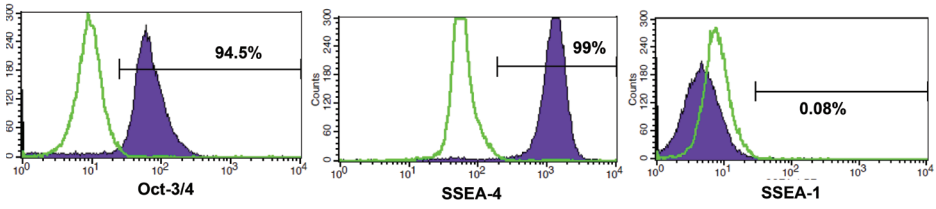
Clump Passaging on Corning Matrigel Matrix (10 passages)

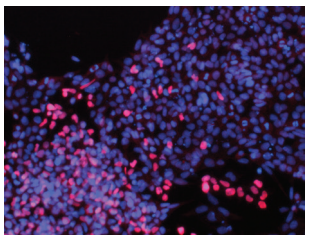


Morphology

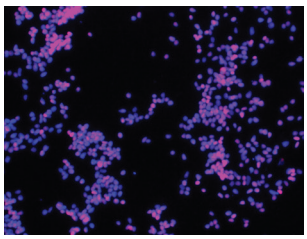


Karyotype





Endoderm (SOX17)



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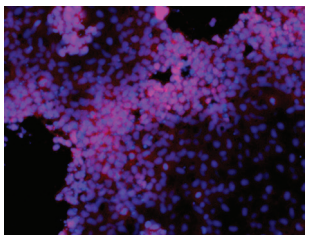


Mesoderm (Brachyury)

MATERIALS AND METHODS

Matrigel coated plates were prepared using Corning Matrigel hESC-qualified matrix (Cat. No. BD354277). Corning PureCoat rLaminin-521 cultureware (Cat. No. 10861-076) was used according to manufacturer's guidelines. Human episomal iPSCs (Gibco®) were routinely maintained on Matrigel coated 6-well plates in mTeSR®1 medium (STEMCELL Technologies™)

On Matrigel coated 6-well plates, cells were clump passaged every three to five days using Cell Dissociation Buffer and seeded at a split ratio of 1:14–1:21 in NutriStem XF/FF medium (manufactured by Biological Industries).

Cells were seeded as single cells onto rLaminin-521 cultureware (6-well format) in NutriStem medium. Cells were passaged every four to five days using Accutase® and seeded at a density of 50,000 cells/cm^2

At each passage, the morphology of the cells and the cell yields (PureCoat only) were monitored. After ten passages, cells were karyotyped at Cell Line Genetics® (WI), expression of Oct-3/4, SSEA-4, and SSEA-1 was evaluated using flow

CORNING

cytometry (BD FACSCalibur™) and directed differentiation into three germ layers was performed using human pluripotent stem cell functional identification kit (R&D Systems); nuclei were counterstained with Hoechst 33342 (blue).

RESULTS

Cell morphology: Using NutriStem, cells displayed typical hPSC morphology on both surfaces.

Undifferentiated marker expression: hiPSCs expressed undifferentiation markers (Oct-3/4 and SSEA-4); differentiation marker (SSEA-1) was not detected.

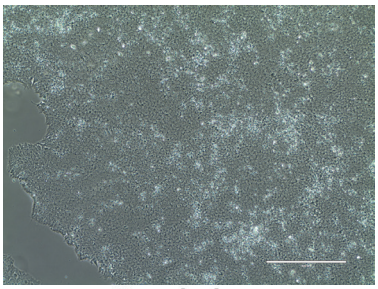
Maintenance of pluripotency: hiPSCs were able to differentiate into three germ layers.

Karyotype: hiPSCs retained a normal karyotype.

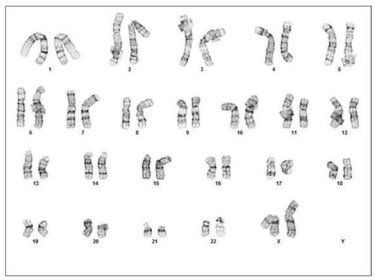
CONCLUSIONS

The xeno-free medium, NutriStem, allows usage of multiple surfaces and passaging methodologies for long-term culture and expansion of hPSCs; clump passaging on Matrigel matrix; and single cell passaging on PureCoat rLaminin-521 cultureware.

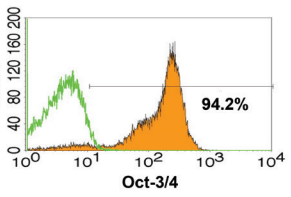
Single CellPassaging on Corning PureCoat rLaminin-521 (10 passages)

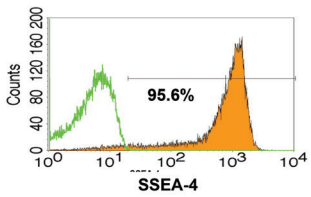


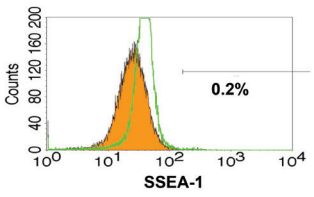
Morphology

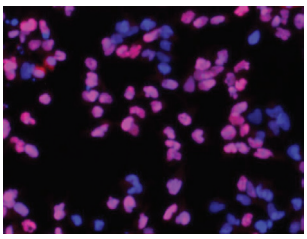


Karyotype

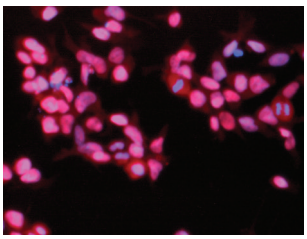




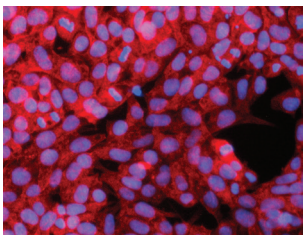




Endoderm (SOX17)



Ectoderm (otX2)



Mesoderm (Brachyury)

Description	Size, mL	Cat. No.	Unit
Corning NutriStem XF Medium	100	75838-638	Each
Corning NutriStem XF Medium	500	75838-636	Each

Mesenchymal Stem Cell (MSC) Research —

Why Culture Conditions and Rigorous Validation of Cell Identity Matter

PromoCell

INTRODUCTION

MSCs are fibroblastoid multipotent adult stem cells with a high capacity for self-renewal. These cells have been isolated from several human tissues, including bone marrow, adipose tissue, umbilical cord matrix, tendon, lung, and the periosteum[2]. MSCs have recently been shown to originate from the perivascular niche, a tight network present throughout the vasculature of the body. These perivascular cells lack endothelial and hematopoietic markers, such as CD31, CD34, and CD45, but express CD146, PDGF-R β, and alkaline phosphatase[3].

According to recent analyses, billions of dollars are spent each year in the United States alone on preclinical research that is not reproducible[1]. The study design, biological reagents and reference material used are all key contributing factors to the lack of reproducibility in preclinical experiments. In the field of mesenchymal stem cell (MSC) research, the impact of misidentification of cell types on experiment reproducibility, and the associated costs, cannot be overestimated. Investigators use different methods for cell isolation and expansion, as well as different approaches to characterize the cells. Using validated research materials and technologies, and adopting the best methodological practices, will lead to substantial improvements in the reproducibility of fundamental and preclinical research, accelerating progress in the field and in the subsequent development of clinical therapies.

Due to the extensive therapeutic potential of human MSCs in the field of regenerative medicine, there has been increasing interest in utilizing these cells in a variety of biomedical disciplines. Several different methods of isolation and expansion have been reported, making it increasingly difficult to compare and contrast study outcomes. Standardization of the methodology is required for reproducibility and facilitating exchange of data towards therapeutic applications. With this in mind, the International Society for Cellular Therapy (ISCT) has defined criteria to ensure the integrity and unambiguous identification of human multipotent MSCs[4]. According to these guidelines, MSCs must express the surface markers CD73, CD90, and CD105 and stain negative for expression of CD14 or CD11b; CD34; CD45; CD79α or CD19; and HLA-DR. In addition, MSCs must differentiate into adipocytes, osteoblasts, myocytes, and chondrocytes *in vitro* (Figure 1).

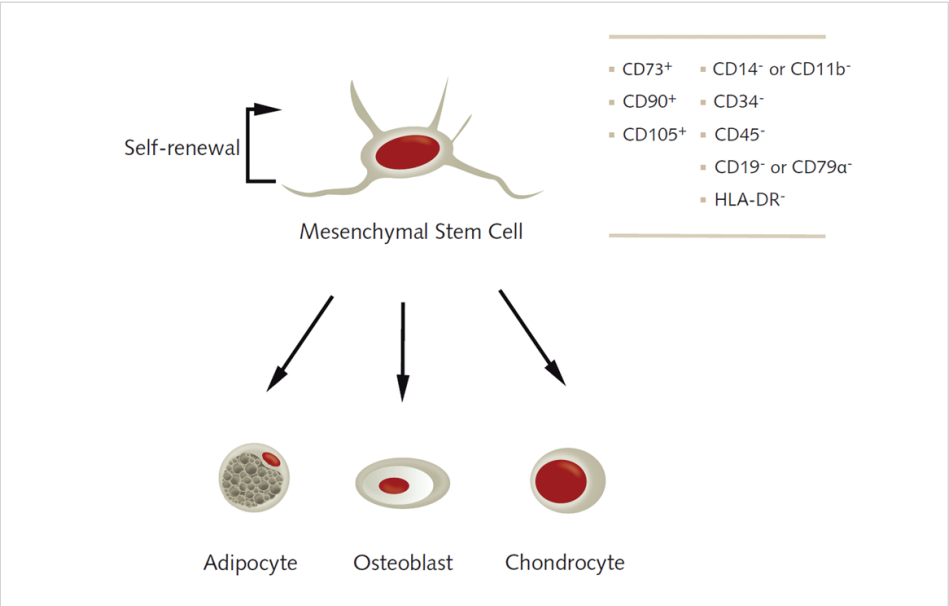


Figure 1. Minimal criteria for defining multipotent human mesenchymal stem cells (MSCs) as proposed by the International Society for Cellular Therapy (ISCT). Human MSCs must express CD105, CD73 CD73, and CD90, and lack expression of CD45; CD34; CD14 or CD11b; CD79α or CD19; and HLA-DR surface molecules. In addition, MSCs must be plastic-adherent when maintained in standard culture and have the ability to differentiate into adipocytes, osteoblasts, and chondrocytes *in vitro*.

RESULTS

Characterization of MSC surface markers by flow cytometry, following isolation from bone marrow using PromoCell's MSC Growth Medium, showed a defined population positive for the markers CD73, CD90, and CD105, and negative for CD14 or CD11b; CD34; CD45; CD79α; or CD19; and HLA-DR (Figure 2). This is in agreement with the ISCT criteria. Further expansion of this cell population using PromoCell MSC Growth Medium DXF on fibronectin-coated tissue culture flasks over the course of 7 serial passages showed a stable growth performance, with an average of 28 hours per population doubling (Figure 3). By passage 7, an expansion by a factor of 4.29 billion was achieved.

Differentiation of expanded bone marrow MSCs into adipocytes, chondrocytes, and

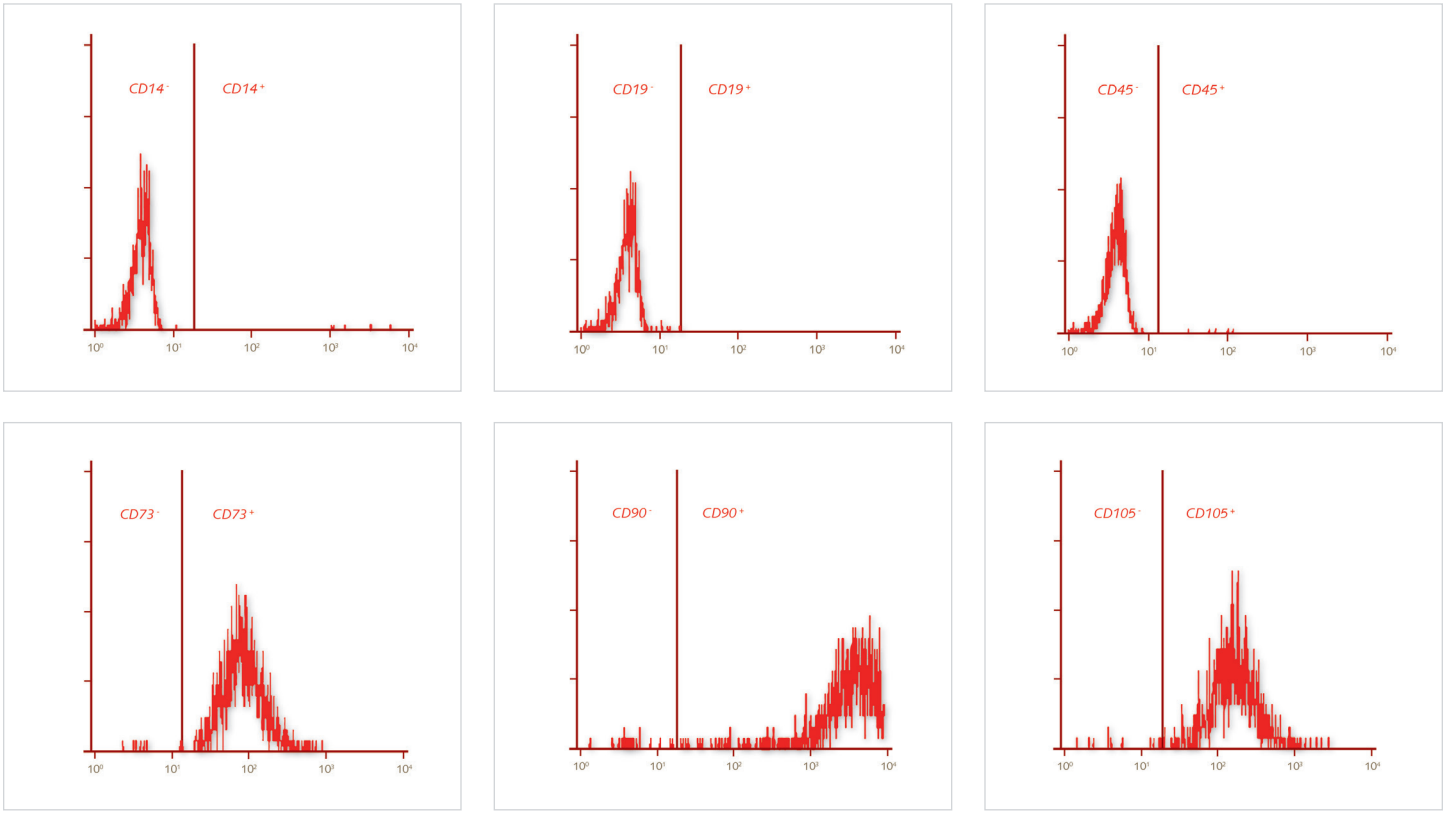


Figure 2. Flow cytometry analysis of PromoCell human primary mesenchymal stem cells isolated from bone marrow. Cryopreserved cells were tested after thawing and show a well-defined population positive for the markers CD73, CD90, and CD105 and negative for CD14 or CD11b; CD34; CD45; CD79α or CD19; and HLA-DR, in agreement with ISCT guidelines.

osteoblasts, in accordance with ISCT criteria, was assayed in passage 3 using PromoCell MSC differentiation media (Figure 4). All of the MSCs tested differentiated successfully into the three cell types, demonstrating their multipotency. Adipogenic differentiation showed extensive intracellular lipid vacuole formation typical of mature adipocytes (Figure 4A). Chondrogenic differentiation of MSCs was determined by induction of cartilage spheroid formation (Figure 4B). Finally, differentiation of MSCs into mature osteoblasts was demonstrated by Alizarin Red S staining of extracellular calcium deposits in mineralized cells (Figure 4C).

Taken together, these data illustrate the isolation and uniform characterization of MSCs allowing comparability of results across the research community.

CONCLUSIONS & PERSPECTIVE

Isolation of human MSCs using PromoCell Media yielded a uniform, well-defined MSC population in agreement with marker expression profiles proposed by the ISCT. The stable growth performance and expansion over the course of several passages demonstrate the self-renewing capabilities of these cells. In addition, multipotency could be demonstrated by differentiation of expanded MSCs into adipogenic, chondrogenic, and osteogenic linages. When considered together, these data indicate that validation of cellular identity based on the ISCT recommendations allows for a well-defined MSC population suitable for *in vitro* expansion while retaining stemness.

The MSC isolation method, especially the culture medium utilized for isolation of cells from tissue, plays a critical role in obtaining a well-defined MSC population

PromoCell

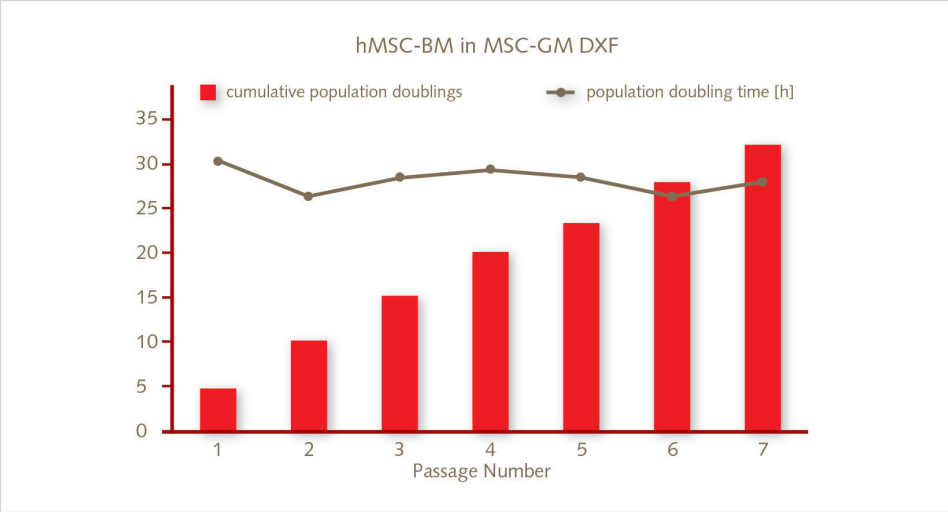


Figure 3. Growth performance of PromoCell human primary mesenchymal stem cells (MSCs) isolated from bone marrow cultured in PromoCell MSC Growth Medium DXF on fibronectin-coated tissue culture plastic. The MSCs used in this investigation all meet the International Society for Cellular Therapy (ISCT) criteria. The cumulative number of population doublings and doubling time is shown over the course of seven passages. A stable growth rate of under 30h/doubling can be observed even after prolonged *in vitro* culture for 32 population doublings over the course of 7 passages.

that is in agreement with the definition included in the ISCT guidelines. As a result, all MSC populations that conform to ISCT- defined marker expression profiles showed sustained proliferation and good differentiation performance, in comparison with cells that did not conform to ISCT criteria, which showed limited proliferative and differentiation potential.

Lastly, the use of MSCs from a well-validated source is increasingly becoming a requirement for funding of preclinical research. Notably, the National Institutes of Health (NIH) has recently updated its approval process and review criteria to ensure that they are funding the most rigorous and robust research in order to enhance reproducibility of research findings through increased scientific rigor and transparency[5].

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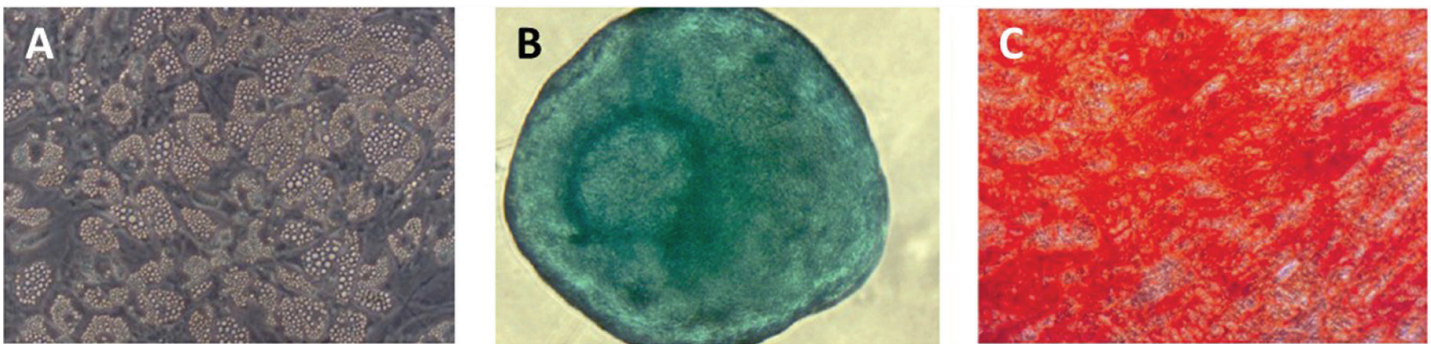


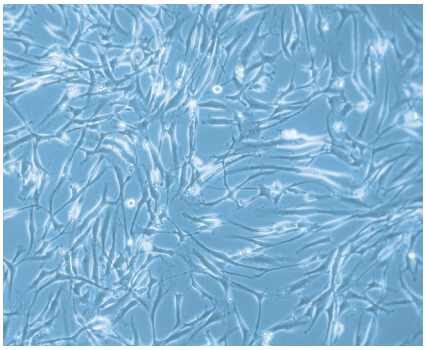
Figure 4. Differentiation of *in vitro* cultured PromoCell human mesenchymal stem cells (MSCs) into adipocytes **(A),** chondrocytes **(B),** and osteoblasts **(C)**. According to the International Society for Cellular Therapy (ISCT) criteria, MSCs must differentiate into all three cell types independent of their origin. **(A)** Lipid vesicle accumulation in adipocytes differentiated from human MSC derived from bone marrow (hMSC-BM) using the PromoCell MSC Adipogenic Differentiation Medium 2. The differentiated culture exhibits extensive intracellular lipid vacuole formation typical of mature adipocytes (100X magnification). **(B)** MSC spheroids after *in vitro* differentiation into cartilage using PromoCell MSC Chondrogenic Differentiation Medium (stained for aggrecans using Alcian Blue). Induced spheroids exhibit an intensely blue color indicative of cartilage extracellular matrix. **(C)** Alizarin Red S staining of extracellular calcium deposits in mineralized hMSC-BM -derived mature osteoblasts. Cells were cultured for 12 days in PromoCell MSC Osteogenic Differentiation Medium.

Mesenchymal Stem Cell and Media System

PromoCell

Reliable Results for Your Research

- **Defined Cells.** Each MSC batch is tested in accordance with ISCT* recommendations for CD73+/CD90+/CD105+ and CD14-/CD19-/CD34-/CD45-/HLA-DR
- **Guaranteed Performance.** Each of our MSC lots is guaranteed to grow with a doubling time of <30h through at least 10 population doublings and are also tested for differentiation into osteoblasts, adipocytes, and chondrocytes
- **Complimentary Research Tools.** PromoCell offers a comprehensive portfolio of cell biology research products including Growth & Differentiation Factors, Stem Cell Fate Regulators, Antibodies & ELISAs, Cellular Analysis, and Cell Transfection



PromoCell is a premier manufacturer of cell culture products with a complete portfolio featuring human Mesenchymal Stem Cell (hMSC) culture media, growth media, and differentiation media. All of our hMSC are isolated in accordance to the highest ethical standards and extensively quality tested including marker characterization, growth performance, and *in vitro* differentiation in our media.

* ISCT (International Society for Cellular Therapy) Cytotherapy (2006) Vol. 8, No. 4, 315-317

Description	Size	Cat. No.	Unit
Human Mesenchymal Stem Cells			
MSC from Bone Marrow (hMSC-BM)	500.000 cryopreserved cells	**10172-152**	**Each**
MSC from Umbilical Cord Matrix (hMSC-UC)	500.000 cryopreserved cells	**10172-148**	**Each**
MSC from Adipose Tissue (hMSC-AT)	500.000 cryopreserved cells	**10172-156**	**Each**
Mesenchymal Growth Media			
MSC Growth Medium 2 (serum containing)	500 mL	**75812-652**	**Each**
MSC Growth Medium DXF (defined & xeno-free)	500 mL	**10172-384**	**Each**
MSC Differentiation Media			
MSC Adipogenic Differentiation Medium 2	100 mL	**75812-654**	**Each**
MSC Osteogenic Differentiation Medium	100 mL	**10172-378**	**Each**
MSC Chondrogenic Differentiation Medium	100 mL	**10172-376**	**Each**

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- Dual temperature probes for over-temperature protection and TC CO_2 sensor for long lasting, reliable performance
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Volume, L (cu. ft).	Cat. No.	Unit
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- Footrest provides support for user's feet and legs

Vinyl upholstered, ergonomic chairs for use at desk, medium-bench and high-bench heights that include seat and backrest tilt, dual-wheel casters, footrings (on medium and high heights), and backrests with lumbar curve. Available with or without armrests. High-bench height, urethane stool includes adjustable footring. All steel footrest provides needed support for legs and feet.

Description	Footring	Seat Height	Cat. No.	Unit
VWR® Contour™ Deluxe FFAC Lab Chairs with Arms				
Desk Height	No	43.2–55.9 cm (17–22")	**10147-207**	**Each**
Medium Bench Height	50.8 cm (20") dia.	53.3–71.1 cm (21–28")	**10147-209**	**Each**
High Bench Height	50.8 cm (20") dia.	53.3–78.7 cm (21–31")	**10147-211**	**Each**
VWR® Contour™ Deluxe FFAC Lab Chairs				
Desk Height	No	43.2–55.9 cm (17–22")	**10152-753**	**Each**
Medium Bench Height	50.8 cm (20") dia.	53.3–71.1 cm (21–28")	**10152-755**	**Each**
High Bench Height	50.8 cm (20") dia.	53.3–78.7 cm (21–31")	**10152-745**	**Each**
VWR® Ergonomic Footrest				
Adjustable, Free-Standing Footrest		—	**97035-500**	**Each**
VWR® Contour™ Self-Skinned Urethane Stool				
High Bench Height	50.8 cm (20") dia.	48.2–73.7 cm (19–29")	**80086-433**	**Each**

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Size	Cat. No.	Case of
Cryo.s Sample Tubes with Datamatrix		
1–1.2 mL, Internal Thread	**89176-676**	**500**
1.8–2.0 mL, Internal Thread	**89176-674**	**500**
up to 2.2 mL, External Thread	**89176-678**	**500**
Accessories for Cryo.s with Datamatrix Storage Rack Lid	**89176-314**	**20**
Racks with 96 Cryo.s Biobanking Tubes, Datamatrix Coded		
300 µL	**75832-302**	**960**
600 µL	**75832-304**	**960**
1000 µL	**75832-306**	**960**

CRISPR/Cas9-Mediated Gene Disruption Using jetPRIME®

Guillaume Freund[A], Alengo Nyamay'Antu[1], Dominique Desplancq[B], Etienne Weiss[B]
A Polyplus-transfection®, 850 Bld Sébastien Brant, 67400 Illkirch, France
B ESBS, UMR 7242, CNRS/Université de Strasbourg, 300 Bld Sébastien Brant, 67412 Illkirch, France

INTRODUCTION

The use of the CRISPR/Cas9 technology in mammalian cells has recently emerged as a very convenient way to modify the cell genome at a specific locus. The CRISPR/Cas9 system is a prokaryotic immune mechanism that confers resistance to foreign genetic elements and consists of a DNA endonuclease, Cas9, and a non-coding guide RNA (gRNA). The gRNA guides Cas9 to a specific complementary genomic locus to introduce a double-strand break.

The success of CRISPR genome editing is limited by the intracellular delivery and expression of Cas9 protein and gRNAs. The most common strategy involves transient transfection into mammalian cells of one or several plasmids coding for Cas9, the specific gRNA, and, if needed, the sequence to be inserted.

Here we show that efficient plasmid delivery with low DNA amounts and low toxicity can be achieved with the Polyplus-transfection® reagent, jetPRIME for successful CRISPR/Cas9-mediated gene knock-out as illustrated for two genes encoding essential proteins involved in DNA replication (PCNA and CDC45).

MATERIAL & METHODS

Cell Culture and Transfection

HeLa cells were maintained in Dulbecco's Modified Eagle's tissue culture medium supplemented with 10% heat-inactivated fetal calf serum at 37°C in a humidified, 5% CO_2 atmosphere. All transfections were performed in 12-well plates (Greiner Bio-One) at a plating density of 10^5 cells per well 24h before transfection. Transfection of HeLa cells was performed with jetPRIME (Polyplus-transfection) using 0.8µg DNA and 1.6µL reagent per well.

CRISPR/Cas9 Assay

Cells were transfected with a single plasmid encoding for Cas9 and a gRNA sequence to target either PCNA or CDC45 gene with a puromycin resistance cassette (Fig 1). Two days after transfection, cells harboring the Cas9-encoding vector were selected using 2µg/mL puromycin for 4 days. HeLa cells were observed with optical microscopy to verify the proper knockout of the PCNA or CDC45 gene (Fig 2A).

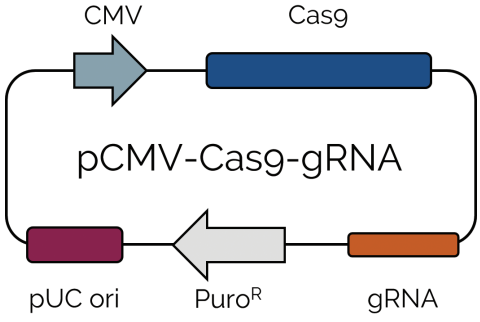

Fig 1. Schematic map of the pCMV-Cas9-gRNA plasmid.

Polyplus transfection®

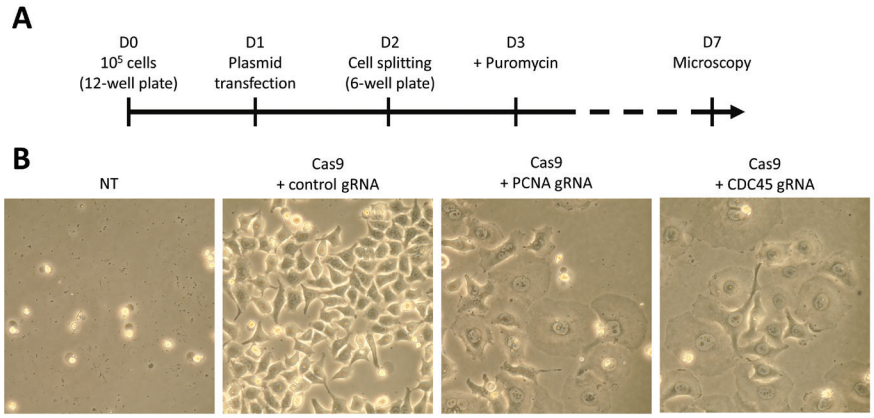

Fig 2. CRISPR/Cas9 gene disruption assay. **(A)** Schematic overview of the CRISPR/Cas9 assay. **(B)** Cell morphology after PCNA or CDC45 gene disruption with CRISPR/Cas9 technology. HeLa cells were transfected with pCMV-Cas9-gRNA plasmid using jetPRIME. 48h post-transfection, the cells expressing Cas9 were selected using puromycin over 4 days. Cells transfected with empty vector are also shown (NT). The pictures show typical fields of the cells observed 6 days post-transfection at the same magnification (400x).

RESULTS

Blockage of DNA synthesis leads to extensive DNA damage such as double-strand breaks (DSBs). It has been reported that cells subjected to inhibition of DNA duplication are affected by phenotypical changes such as nuclear size and cytoplasm enlargement before subsequent cell death. Moreover, proteins involved in DNA synthesis like PCNA (processivity factor) and CDC45 (helicase cofactor) have been identified as markers of replication stress[1,5].

To investigate whether knock-out of PCNA or CDC45 gene can be achieved using the CRISPR/Cas9 method, HeLa cells were transiently transfected with pCMV-Cas9-gRNA following the jetPRIME protocol. Upon puromycin selection, transfected cells were observed with optical microscopy (Fig 2B).

Co-expression of Cas9 and gRNA targeting either PCNA or CDC45 gene clearly affected the morphology and viability of puromycin-selected HeLa cells. Microscopy analysis six days post-transfection showed cell flattening and increased nuclear size in comparison to cells transfected with a plasmid expressing Cas9 and control gRNA. These phenotypical changes suggest efficient gene knock-out of PCNA and CDC45 gene achieved with the CRISPR/Cas9 genome editing method.

CONCLUSION & PERSPECTIVES

Developing an easy and straightforward method for efficient delivery of one or multiple plasmid DNA coding for Cas9 and a gRNA of interest has become indispensable for the *in vitro* use of CRISPR/Cas9 technology. Here, we demonstrated that the transfection reagent jetPRIME is very efficient in delivering DNA plasmid encoding Cas9 and gRNA of PCNA or CDC45, using small amounts of DNA while respecting cell morphology and viability. Moreover, jetPRIME is suitable for multiple plasmid co-transfection which makes it the perfect solution for a variety of developed plasmid DNA-based CRISPR/Cas9 kits. Accordingly, several groups have already successfully used jetPRIME-mediated transfection of the CRISPR/Cas9 system as illustrated by their respective publications[2,3,4].

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Description	Reagent Size, mL	Buffer Size, mL	Cat. No.
jetPRIME® Transfection reagent	0.1	5	**89129-920**
	0.75	60	**89129-922**
	1.5	2 x 60	**89129-924**
	5 x 1.5	10 x 60	**89129-926**
	5 x 1.5	120 (5X Concentrated)	**89137-972**

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BD Peptone Starter Paks provide samples of our most widely used peptones tailored for specific applications, including monoclonal antibodies, recombinant proteins, and vaccines.

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Description	Cat. No.	Unit
BD Peptone Starter Pak No. 1	75796-502	Each
BD Peptone Starter Pak No. 2	75796-504	Each
BD Peptone Starter Pak No. 3	75796-506	Each

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Sterile Polystyrene Daily Shift Head, Contact Plate		75804-756	Each
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Sterile Polystyrene Daily Shift Head, Petri Plate		75804-758	Each

Digital Imaging for Western Blots:

Why It's Essential and What to Look for in a Digital Imaging System

The advent of digital imaging has transformed Western blot detection. Here we outline how Western blotting has changed, and review qualities to look for in a digital imager.

Chemiluminescence and Fluorescence

When most people think of imaging their Western blot, they think of going into a darkroom and exposing the blot to film to detect a chemiluminescent signal. A much more powerful alternative is digital imaging, which in the last decade has seen radical advancements of technology, leading to improved speed, sensitivity, and quantitative data when detecting chemiluminescence. This technology has also opened the door to other options such as fluorescent detection for imaging and quantifying Western blots.

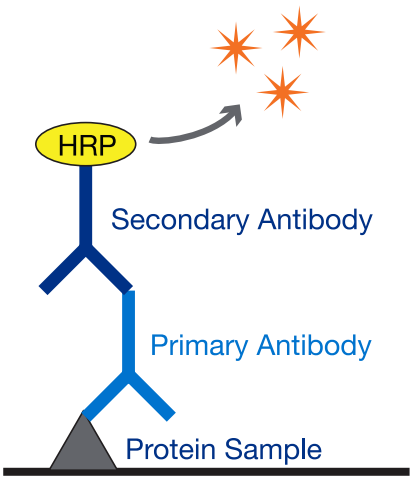

Figure 1. Chemiluminescent Western Blotting. In chemiluminescent detection, the antigen-primary antibody complex is bound by a secondary antibody conjugated to an enzyme, such as horseradish peroxidase (HRP), that generates light in the presence of a luminescent substrate. The light can be detected by exposure to X-ray film or by CCD imaging systems.

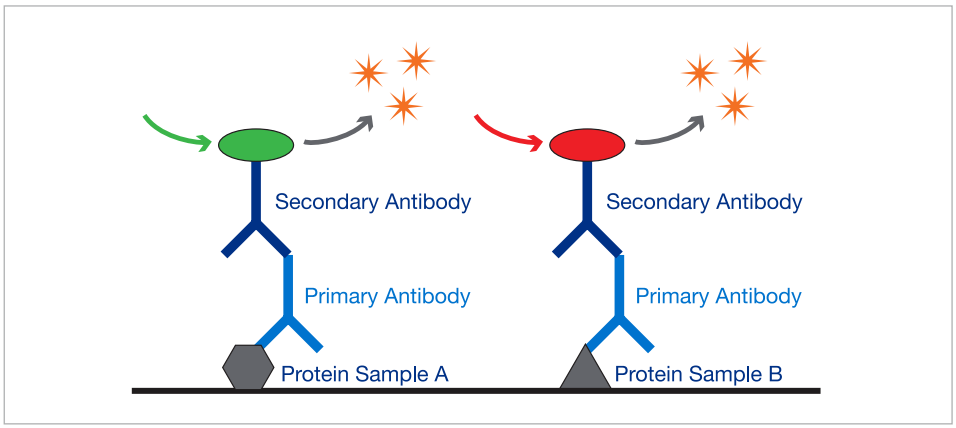

Figure 2. Fluorescent Multiplex Western Blotting. In fluorescent detection, the antigen-primary antibody complex is bound by a secondary antibody conjugated to fluorescent dye. The fluorescence of the dye can be detected by a digital camera in an imaging system equipped with the correct light source to excite the dye and the correct filters to detect the emitted light. Multiplex detection is possible by using two different fluorescent dyes and an instrument that can excite and detect the light from each.

Chemiluminescence is a popular detection method for Western blotting because of its inherent sensitivity[3] (Figure 1). This technique is a well-established way to detect one protein in a sample. Chemiluminescence is very good at answering the question, "Is my protein there or not?" but it is not very good at addressing other important questions, such as: How much of my protein is present relative to another protein? How much of my protein is in one sample compared to another sample? How do I control for sample loading inconsistencies?

While chemiluminescence has become the method of choice for one signal, one-protein detection[1], the recent introduction of multiplex fluorescence has allowed people to use the spectral properties of fluorescent probes to obtain multiple signals from multiple proteins on one blot[2]. In fluorescent detection, the secondary antibody is coupled to a fluorophore with known emission and excitation wavelengths (Figure 2). Multiple secondary antibodies can be detected (multiplex detection) if each antibody is conjugated to a different fluorophore having different spectral characteristics.

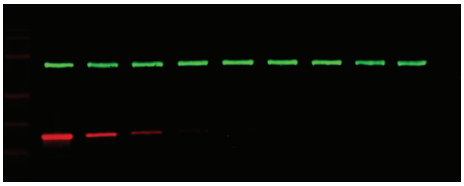

Figure 3. Loading controls enable accuracy when comparing multiple samples. Transferrin was probed for in the 800 channel, while GPDH was probed for in the 700 channel.

Why Mutiplex?

Why examine more than one protein on a blot? The use of loading controls is a common reason (Figure 3). Loading controls are usually "housekeeping genes" — proteins that exhibit high-level, constitutive expression in the cell type or sample you are examining and are expected to be present at similar levels in all samples. To control for inconsistencies in the amount of protein between lanes on a gel, the signal from the protein of interest is divided by the signal from the loading control for each lane. Using a loading control allows you to compare the

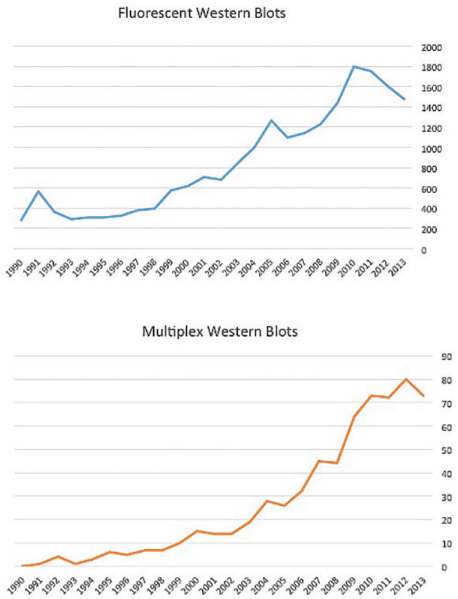

Figure 4. Publication Trends in Western Blotting. Over the past 23 years, the number of publications reporting the use of fluorescent Western blots and of multiplex Western blots has increased tremendously.

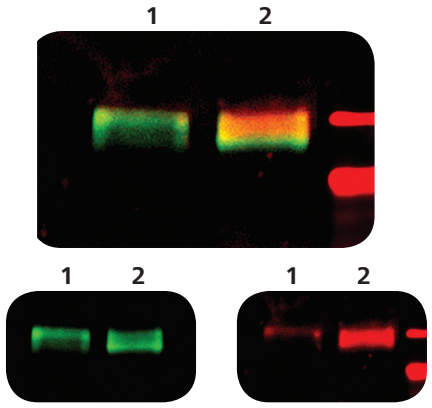

Figure 5. Simultaneous detection of EGFR and phospho- EGFR. Control cells (lane 1) and cells treated with EGF (lane 2) were imaged. EGFR was detected in the green channel (panel B), and phospho-EGFR was detected in the red channel (panel C). Panel A shows the green and red channels superimposed.

amount of protein of interest between samples and have increased confidence that any differences are real.

With chemiluminescence, it is possible to probe for more than one protein when the proteins have different molecular weights and are spatially separated on the blot. However, your protein of interest might not be well separated from your loading control. And, if you probe simultaneously for two different proteins with chemiluminescence, you might mistakenly think a degradation product of the larger protein is the smaller protein, or a multimer of the smaller protein is the larger protein, if the modified products run near the expected molecular weight. For this reason, chemiluminescence is not ideal for multiplexing, even when the two target proteins are different sizes.

Another reason to look at multiple proteins on a single blot is to study post-translational modifications such as phosphorylation, methylation, and glycosylation. Many posttranslational modifications do not significantly change the molecular weight of a protein so the unmodified and modified isotypes migrate too closely together to examine using chemiluminescence.

Stripping the primary and secondary antibodies off of a blot and re-probing the blot with a second set of antibodies is an option to detect a second protein using chemiluminescence. However, the most common stripping protocols, in which the blot is incubated with a heated solution containing detergent and a reducing agent, can remove sample protein from the membrane and may not completely strip the first set of antibodies[4]. Often the second detection is "dirty", with background noise that reduces sensitivity. And, if the second target protein has a similar molecular weight to the first one, as is the case for many post-translationally modified proteins, there can be a concern that some of the signal observed after re-probing is leftover signal from the first probing. The procedure takes time, could affect your results, and results never are as clean as a fresh blot. A better alternative is needed. Fluorescence detection is a superior method for multiplex detection. The numbers of publications reporting the use of fluorescent Western blots, and of multiplex Western blots, have increased substantially over the past 20 years (Figure 4). Multiplex fluorescent Westerns now allow scientists to probe for multiple proteins at once without stripping and re-probing, and to get clear results regardless of the physical separation of the proteins on the gel (Figure 5).

Get Out of the Darkroom

Most scientists still use a darkroom to process film images of their chemiluminescent Western blots. Film is well known, it's easy, it's sensitive — why change? Film is expensive and requires toxic chemicals. Film also has a small dynamic range and is quickly saturated, so film exposures are not good for quantitative comparisons of chemiluminescent signals. Digital imaging provides a much larger dynamic range so low- and high-intensity bands can be imaged simultaneously. Also, digital imaging produces a file that is immediately ready for publication, while sheets of exposed film must be photographed or scanned to generate a digital image.

Additionally, film can't image multiplex fluorescence. Only digital imaging systems give you this capability.

Important Characteristics to Look for When Purchasing a Digital Imager

High Resolution. High-resolution CCD cameras enable you to see fine detail in your image when you zoom in. The larger the number of pixels, the greater the resolution.

azure biosystems

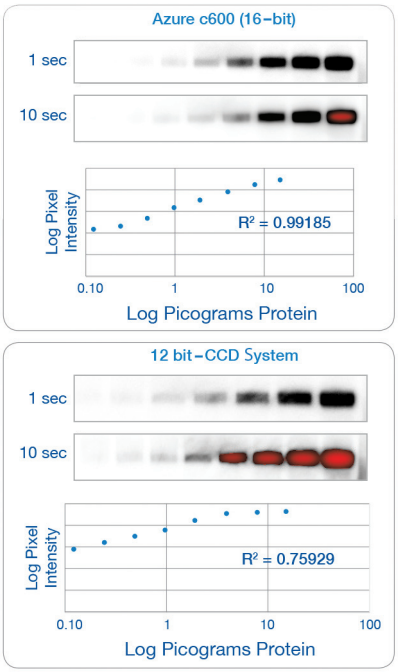

Figure 6. 16 bit Imaging for a Wide Dynamic Range. A Western blot was imaged on both the Azure c600 (a 16-bit system) and a competitive 12-bit system. While the 10 second exposure appears similar on the different systems, the 12 bit system produces an image that is saturated, and not suitable for analysis.

Wide Dynamic Range. Dynamic range is themeasure of signal to noise of a system. Without a wide dynamic range, your strong signals will saturate before you can detect your weak bands, making quantitation impossible (Figure 6). The F stop is an important value to consider, especially when using chemiluminescence. The smaller the F stop, the wider the aperture, and the more light that can be let in. Small F stops drastically reduce exposure times.

Dye Flexibility. The availability of compatible dyes is also important to those who want to do multiplex fluorescent Westerns. You will want to look at a system that has multiple excitation sources and multiple detection wavelengths to be compatible with a wide range of dyes.

Additionally, you will want to make sure that the best light sources have been selected. For example, IR dyes are typically excited with lasers, in part because lasers only emit light close to the excitation peak of the dye. Check to make sure the instrument is able to excite and detect the dyes you work with.

Imaging Flexibility. Many Western blot imaging systems also offer DNA and protein gel documentation, through such features as dualwavelength UV transilluminators, white lights, and blue lights. This allows labs to image their routine DNA gels and protein gels stained with common fluorescent and colorimetric stains.

Sensitivity. Another important feature is the sensitivity of the instrument. If you request a demo of the machine, what is the limit of detection? Does it meet your needs?

Ease of Use. Finally, only at a demo can you really address the ease of use. While "ease of use" might not sound important, think about the future. Do you want to have to train every new person in the lab on the instrument, or do you want a system that they can figure out by themselves?

In summary, digital imaging provides benefits for both chemiluminescent and fluorescent Western imaging. When choosing a digital imaging system, specs are important, but also test the system yourself to make sure it is well designed and easy to use.

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4. Yeung YG, Stanley ER. A solution for stripping antibodies from PVDF immunoblots for multiple

Manufacturer	Kit
Azure	AzureSpectra Fluorescent Western Blotting Kits
LI-COR	LI-COR® Odyssel Western Blotting Kits
Life Technologies	WesternDot® Fluorescent Immunodetection Kits
ProteinSimple	SpectraPlex Fluorescent Western Blot Kit
Abcam	Optiblot Fluorescent Western Blot Kit
Advansta	WesternBright™ MCF and MCF-IR
Rockland Immunochemicals	MaxTag™ for IRDYE® Immunoblotting
Antibodies online/ Genscript	ONE-HOUR Western Fluorescent Kit

Table 1: Commercially available kits.

Description	Cat. No.	Unit
Azure c600 Imaging System	**10147-214**	**Each**

EDTA-Resistant IMAC Resin Minimizes Ni Leakage with Cell Culture Samples

With a goal of helping researchers achieve better chromatography results and faster runs, scientists at GE Healthcare have strived for decades to continuously develop innovative tools. One example is an IMAC resin used to purify his-tagged proteins from samples containing components that would normally strip the immobilized metal ions from the resin during sample loading. By switching to Ni Sepharose™ excel for these sample types, it is possible to attain efficient and simplified workflows for capture, purification, and reproducible screening of his-tagged proteins secreted into cell culture media. Ni Sepharose excel is compatible with both eukaryotic cells, such as insect and Chinese Hamster Ovary (CHO), and prokaryotic samples, such as *E. coli* extracts.

Cell Culture Media Can Contain Components that Will Affect the Yield

Mammalian (e.g., CHO cells) and insect cell culture media contain substances that might have a negative effect on the purification yield of extracellularly expressed histidine-tagged (his-tagged) proteins. These components lower the binding to conventional immobilized metal affinity chromatography (IMAC) resins (Fig. 1) by stripping the chelated metal ion, which can be visualized by the loss of a nickel-charged resin's blue color. The purification is further complicated by the fact that the target protein concentration in these samples is often low, requiring the use of large sample volumes, which in turn could lead to increased metal ion stripping. To overcome these obstacles, considerable sample pre-treatment is required. Often, buffer exchange by diafiltration in combination with increased concentration needs to be performed before purification. Such pre-treatment is time consuming, potentially harmful to sensitive proteins, and can cause unnecessary loss of target protein.

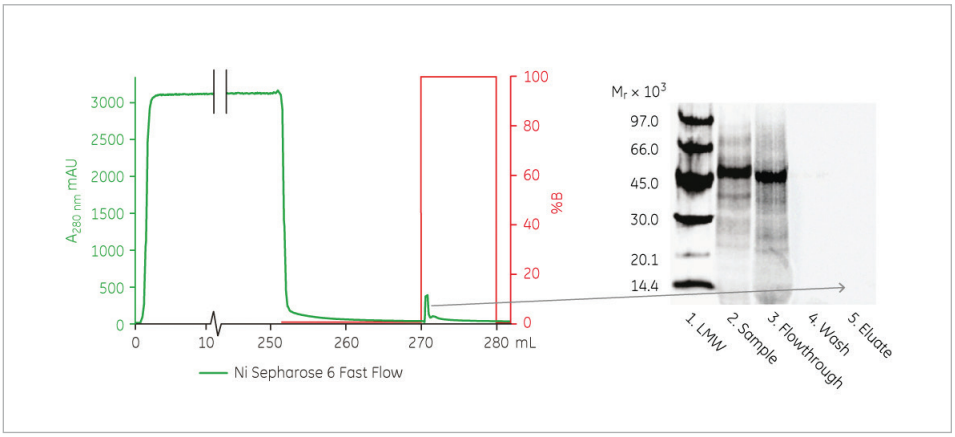

Figure 1. His-tagged protein expressed in CHO cells and extracellularly expressed into CHO cell culture medium was loaded onto Ni Sepharose 6 Fast Flow. SDS-PAGE analysis of the eluate from Ni Sepharose 6 FF showed no recovery of target protein.

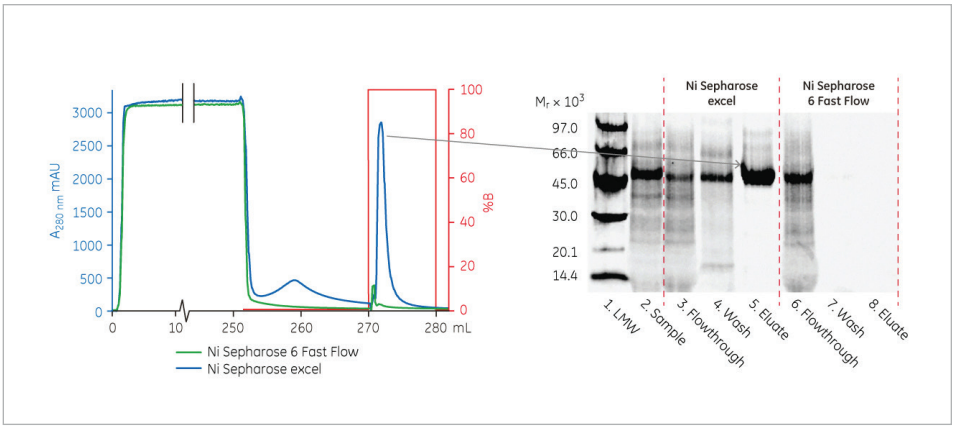

Figure 2. The same sample was loaded onto Ni Sepharose excel. SDS-PAGE analysis showed that the protein could be successfully purified using Ni Sepharose excel, with a purity of > 98%.

Distributor
GE Healthcare

Ni Sepharose excel enables target protein binding in the presence of nickel stripping components

The nickel ions are so strongly bound to this IMAC resin that samples that would normally cause stripping can be loaded onto the resin without pretreatment (Fig. 2). The Ni Sepharose excel resin keeps its blue color, providing additional evidence that Ni Sepharose excel resists nickel stripping in the presence of as much as 100mM EDTA. Because pre-treatment is not needed, Ni Sepharose excel will save you significant time (Fig. 3), while preventing degradation of your sensitive protein. Product formats are shown in Figure 4.

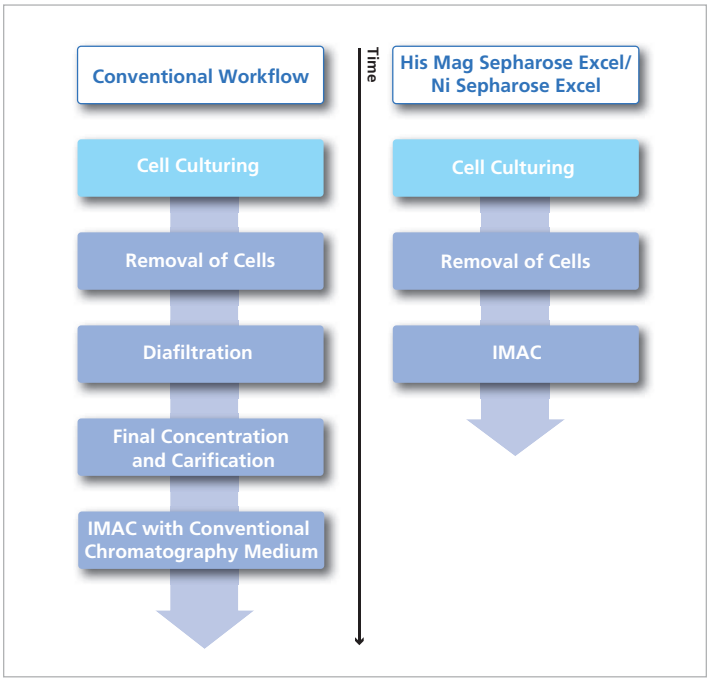

Figure 3. (above) Ni Sepharose excel significantly simplifies and speeds up the workflow compared to conventional IMAC workflows.

Figure 4. (right) His Mag Sepharose excel is composed of magnetic beads designed for simple and efficient small-scale purification and screening. Ni Sepharose excel is available for all scales of work from convenient, prepacked HisTrap excel columns to bulk quantities.

HiTrap™ Protein Purification Columns

Pre-Packed, Ready-to-Use Columns for Fast and Easy Protein Purification

Distributor
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- Comprehensive range of 170 different columns and pre-packed resins to cover the most common chromatrography techniques
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Description	I.D. x L, mm	Cat. No.	Unit
HisTrap Excel Ni			
5 x 1mL, <4mL/min	7 × 25	**89233-412**	**Each**
5 x 5mL, <20mL/min	16 × 25	**89233-414**	**Each**
HiTrap IMAC HP			
5 x 1mL, <4mL/min	7 × 25	**95056-042**	**Each**
5 x 5mL, <20mL/min	16 × 25	**95056-152**	**Each**

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G. Heavy Gauge Hinges:
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J. Leveling Bolt Access at all Four Corners:
Provides access for minor adjustments. Cabinet base features a die-formed gusset with a 3" long leveling bolt.

K. One-Piece Case Bottom and Front Rail:
Bottom is radiused at sides and rear for easy cleaning.

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Prevents dirt and bacteria from collecting in inaccessible areas, and prevents contamination in the laboratory.

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A Novel pH Gradient Separation Platform for Monoclonal Antibody (MAb) Charge Variant Analysis

thermo scientific

Shanhua Lin, Thermo Fisher Scientific Sunnyvale, USA, Julia Baek, Thermo Fisher Scientific Sunnyvale, USA, Srinivasa Rao, Thermo Fisher Scientific Sunnyvale, USA, Yury Agroskin, Thermo Fisher Scientific Sunnyvale, USA, Chris Pohl, Thermo Fisher Scientific Sunnyvale, USA, Wim Decrop, Thermo Fisher Scientific, Amsterdam, The Netherlands,, Dafydd Milton, Thermo Fisher Scientific, Runcorn, UK

ABSTRACT

MAb charge variants and protein standards with various pI values have been successfully separated using cation exchange chromatography with a linear pH gradient. This linear pH gradient is generated by running a linear pump gradient from 100% buffer A (at pH 5.6) to 100% buffer B (at pH 10.2). Ruggedness testing of this pH gradient on Thermo Scientific™ MAbPac™ SCX-10 columns shows that retention time RSD is less than 0.8% over 300 runs.

INTRODUCTION

There is an extensive and increasing development pipeline for monoclonal antibody (MAb) therapeutics, which, combined with advances in automation in upstream processes such as cell culture and purification process development, is driving a requirement for innovative analytical tools in order to deliver greater productivity. Recombinant MAbs can be highly heterogeneous due to modifications such as sialylation, deamidation and C-terminal lysine truncation. During development and production of these products, it is essential to detect, characterize, and quantify impurities as well as structural variants and modifications, and to monitor product stability. This is key to demonstrating their safety and efficacy as biotherapeutics and is required by the U.S. FDA and other regulatory agencies.

Traditionally, cation exchange chromatography using salt gradients has been successfully employed to characterize MAb charge variants. However, additional effort is often required to tailor the salt gradient method for individual MAbs. In the fast-paced drug development environment, a generic platform approach, which saves time in method development and facilitates method transfer for a wide range of MAb charge variants while still using an LC system, is desirable[1].

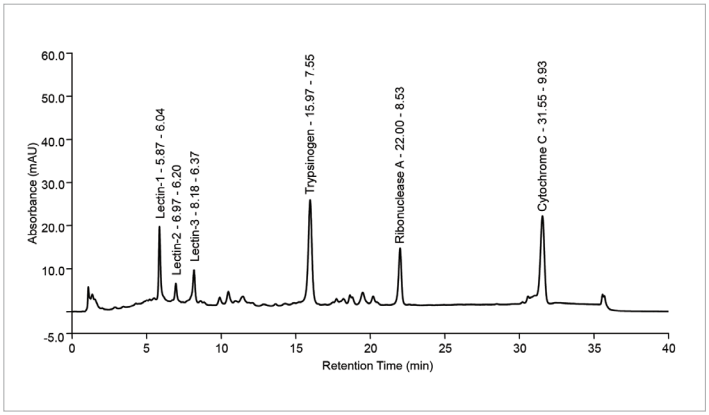

Figure 1. Chromatogram of six proteins separated on a 30 min linear pH gradient on a MAbPac SCX-10, 10µm, 4 x 250mm column. Protein name, retention time, and corresponding pH values are labeled for each protein peak.

In this study, we present a novel pH gradient method for cation exchange chromatography. The linear pH gradient was achieved by employing a multi-component buffer system containing multiple zwitterionic buffer species with pI values ranging from 6 to 10. Eluent A was titrated to pH 5.6 and eluent B was titrated to pH 10.2. In this pH range, each buffer species was either neutral or negatively charged. Therefore, they were not retained by the cation exchange stationary phase and served as good buffers for the mobile phase and the stationary phase.

MATERIALS & METHODS

Consumables

Proteins and MAb were dissolved in deionized water.
Thermo Scientific™ CX-1 pH Gradient Buffer Kit
This kit includes 125mL of 10X buffer A concentrate (pH 5.6) and 125mL of 10X buffer B concentrate (pH 10.2).

Time (Minutes)	Flow Rate (mL/min)	%A	%B
0.1	1	100	0
1-31	1	100-0	0-100
31-34	1	0	100
34-40	1	100	0

Table 1: 30 minute linear gradient method used with the MAbPac SCX-10, 10µm, 4 x 250mm, cation exchange column. Total run time is 40 min. The linear pH range covers from pH 5.6 to pH 10.2.

Time (Minutes)	Flow Rate (mL/min)	%A	%B
0-1	1	100	0
1-16	1	100-0	0-100
16-17	1	0	100
17-20	1	100	0

Table 2: 15 minute linear gradient method used with the MAbPac SCX-10, 5µm, 4 x 50mm, cation exchange column. Total run time is 20 min. The linear pH range covers from pH 5.6 to pH 10.2.

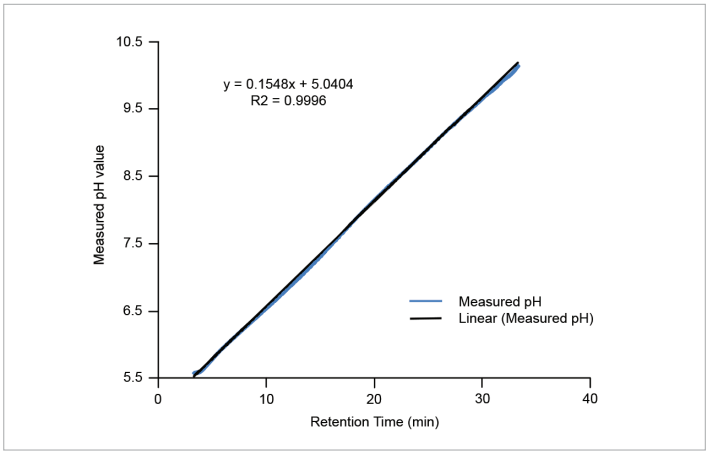

Figure 2. Graph showing measured pH values as a function of time. The measured pH values were exported from the same experiment shown in Figure 1.

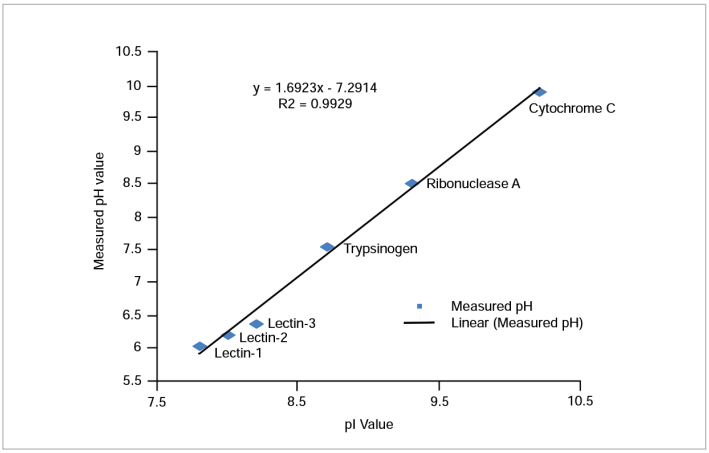

Figure 3. Graph plotting the measured pH values for six protein component peaks as a function of the corresponding pI value. The measured pH values of all six components were exported from the same experiment shown in Figure 1.

Columns
MAbPac SCX-10, 10µm, 4 × 250mm
MAbPac SCX-10, 5µm, 4 × 50mm

Solutions
Eluents A and B each were prepared by diluting the corresponding 10X buffer concentrates 10 fold using deionized water.

Gradients
The linear pH gradient was generated by running from 100% eluent A (pH 5.6) to 100% eluent B (pH 10.2). For pH gradient analysis carried out on the MAbPac SCX-10, 10µm, 4 × 250mm, cation exchange columns, the gradient method in Table 1 (below) was used unless stated otherwise. For pH gradient analysis carried out on the MAbPac SCX-10, 5µm, 4 × 50mm columns, the gradient method in Table 2 (below) was used unless stated otherwise. Both methods cover the pH range from pH 5.6 to pH 10.2.

Data Processing and Software
Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System
The linear gradient is run from 100% buffer A (at pH 5.6) to 100% buffer B (at pH 10.2). Using an online pH meter, it is confirmed that a linear pH gradient was achieved in the range of pH 6 to 10. Furthermore, there is a strong correlation between the measured pH values of model proteins and their pI values. Since the majority of MAbs have their pI values in the range of pH 6 to 10, this pH gradient based separation method can serve as a more generic platform method for MAb charge variant analysis. Once the approximate pH elution range of the target MAb has been established in the initial run, further optimization of the separation can simply be achieved by running a shallower pH gradient over a narrower pH range. Ruggedness testing of this pH gradient on a MAbPac SCX-10, 5µm column has shown that peak retention time RSD is less than 0.8% over 300 runs. Extensive testing of this pH gradient method using various cation exchange columns for charge variant separation of MAbs and other protein samples has demonstrated its capability for fast and high resolution separations.

RESULTS
pH Gradient Linearity
Using the gradient method shown in Table 1, six proteins with a range of pI values from 6 to 10 were effectively separated on a MAbPac SCX-10, 10pm, 4 x 250mm column. These proteins were lectin (including three isoforms, lectin-1, lectin-2, and lectin-3), trypsinogen, ribonuclease A, and cytochrome C. The chromatogram was shown in Figure 1. The pH value measured in this experiment as a function of time was plotted in Figure 2. The pH gradient was found to be linear from pH 5.6 to pH 10.2 over a 30 minute gradient, with a correlation coefficient value R2 of 0.9996 . The correlation between the elution pH for the peaks and the corresponding pI

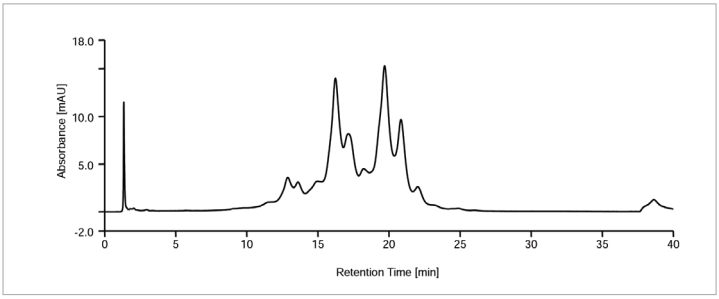

Figure 4. An example of MAb charge variant separation using a salt gradient. The separation was carried out on a MAbPac SCX-10, 10µm, 4 × 250mm column. Eluent A contained 20 mM MES and 60 mM NaCl (pH 5.6) and eluent B contained 20mM MES and 300mM NaCl (pH 5.6). Flow rate was at 0.76 mL/min. A shallow salt gradient was run from 10% B to 30% B from 2 to 32 min, followed by a 2 min wash at 30% B and 1 min wash at 100% B. The column was pre-equilibrated for 15 min at 10% B prior to the gradient. The total run time was 55 min.

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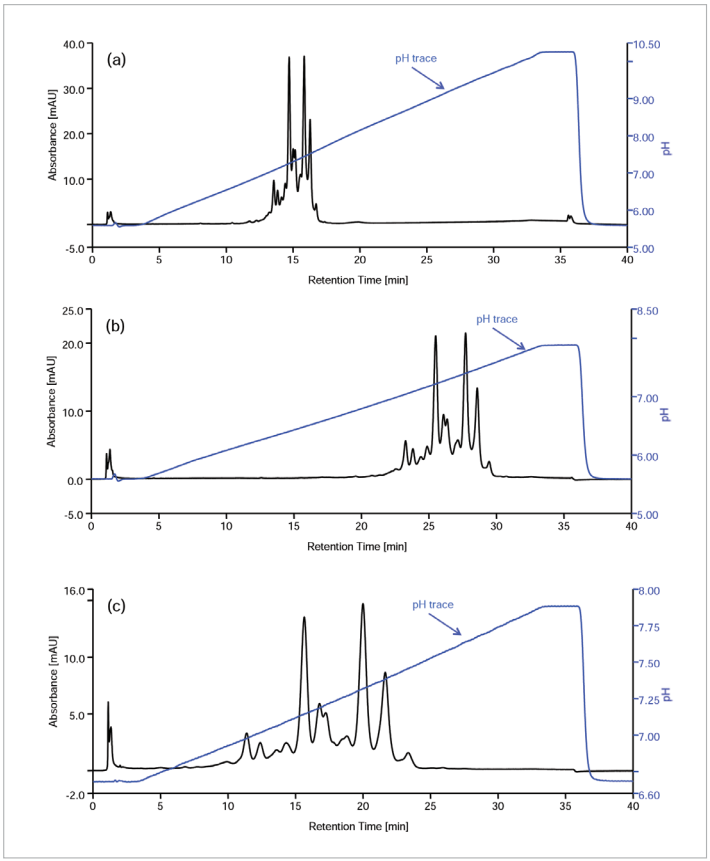

Figure 5. An example ofMAb charge variant separation using a linear pH gradient. The separation was carried out on a MAbPac SCX-10,10 Jm,4 x 250 mm column. (a) Separation by pH gradient,0% B (pH 5.6) to 100% B (pH 10.2) gradient method was shown in Table 1 (b) Separation by pH gradient,0% B (pH 5.6) to 50% B (pH 7.9) (c) Separation by pH gradient,25% B (pH 6.75) to 50% B (pH 7.9)

values of the protein components was assessed. This is shown in Figure 3, which compares the measured pH values for the six protein component peaks shown in Figure 1, as a function of their corresponding pI values. The measured pH values for the six protein component peaks exhibited a strong linear correlation to the literature based pI values. Thus, after a calibration procedure, this example supports the fact that linear regression coupled with the pH gradient method described here can be used to estimate the pI of a protein component based on the peak retention time and measured pH.

Comparison Between pH Gradient and Salt Gradient

Figure 4 shows the separation of a MAb on a MAbPac SCX-10, 10µm, 4 × 250mm column. Using a shallow salt gradient (from 84mM NaCl to 132mM NaCl in 30 min), MAb variant peaks were not very sharp, although they were somewhat separated. In order to further optimize the salt method, it may be necessary to

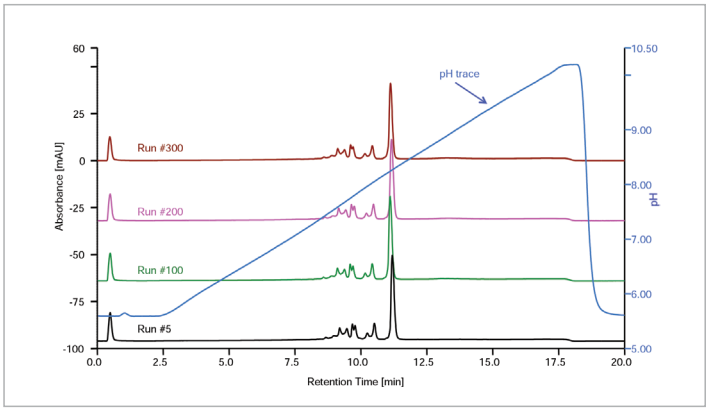

Figure 6. Ruggedness testing of pH gradient on a MAbPac SCX-10,5µm,4 x 50mm column. Gradient method was shown in table 2. The sample was ribonuclease A.

experiment with changing parameters such as buffer salt and pH. Instead of using a salt gradient, a pH gradient approach was used to separate the charge variants.

In the initial run (Figure 5a) the pH elution range from pH 5.6 to pH 10.2 with a gradient slope of 0.153 pH unit/min was chosen. Further optimization of separation can simply be achieved by running a shallower pH gradient over a narrower pH range. Figure 5b shows the separation profile from pH 5.6 to ph 7.9 with pH gradient slope at 0.076 pH unit/min. Figure 5c shows the separation profile from pH 6.75 to pH 7.9 with pH gradient slope at 0.038 pH unit/min. The pH traces in Figure 5a, 5b, and 5c demonstrate that the pH gradient maintains linearity when the slope was reduced to 1/2 or 1/4 of the initial run. The chromatographic profile and therefore the elution order of the variants remained predictable when running a shallower pH gradient. Pump methods for the chromatograms shown in Figure 5b and 5c can be automatically generated by writing a post-acquisition script using the MAb variant pH elution range information collected in the initial run (Figure 5a). This elution predictability demonstrates one of the major advantages of using a pH gradient separation platform, which is to simplify and automate the method development for MAb charge variant separation.

Ruggedness

In addition, the ruggedness of a fast pH gradient method was assessed using a protein standard. Figure 6 shows the elution of ribonuclease A using pH gradient run on a MAbPac SCX-10, 5µm, 4 x 50mm column. The gradient time was 15 min with a total run time of 20 min. The retention time RSD of the ribonuclease A peak was less than 0.8% over 300 runs. This demonstrates the high level of reproducibility with which the pH gradient can be applied to charge variant separations.

CONCLUSION

- A linear pH gradient from pH 5.6 to pH 10.2 can be generated reproducibly using a multi-component zwitterionic buffer system and a MAbPac SCX-10 column.
- The pH gradient can be easily optimized, thereby simplifying charge variant separation method development.
- The pH gradient separation platform enables high resolution, fast and rugged MAb charge variant analysis.

Footnote

HPLC experiments were carried out using a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS System equipped with: SRD-3600 Membrane Degasser, DGP-3600RS Biocompatible Dual-Gradient Rapid Separation Pump TCC-3000SD Thermostated Column Compartment with two biocompatible 10-port valves WPS-3000TBRS Biocompatible Rapid Separation Thermostated Autosampler, VWD-3400RS UV Detector equipped with a micro flow cell PCM-3000 pH and Conductivity Monitor.

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Description	Size, mL	Cat. No.	Unit
CX-1 pH Gradient Buffer Kit	125	10053-802	Each
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Reference

1. Farnan, D, Moreno,T. Multiproduct high-resolution monoclonal antibody charge variant separations by pH gradient ion-exchange chromatography. Anal. Chem. 2009;81:8846-57.

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Best Practices for Life Science Laboratory Design and Redesign

Jessica Burdg, Scientific Journalist

Laboratories are built and existing spaces are redesigned for a number of reasons. Perhaps a space has become outdated, no longer serving the needs of the researchers or their organization. Perhaps additional space is required as a result of personnel growth or procedural requirements. Or, perhaps environmental and efficiency initiatives prompt an overhaul of "business as usual" in how a laboratory functions.

While the reasons are varied, one truth remains: As science evolves and new techniques, tools, and opportunities are presented, many lab managers and safety officers will be faced with life science lab design or redesign questions. What's the best way to navigate what is undoubtedly a complicated, time-consuming process? What is the proper combination of lab layout, equipment, and materials to maximize energy savings, safety, and flexibility? Let's discuss some best practices.

Considerations for Lab Managers and Safety Officers

Matt Anderson, Biosafety Officer at the University of Nebraska-Lincoln, has five points for analysis he uses to determine what makes a lab safe: design, engineering controls, procedures, personal protective equipment (PPE), and people.

- **Design.** While designs vary based on procedural requirements, there are several common components of well-designed labs. For example, specialized research—microscopy, work with radioactive materials, research involving pathogens or high hazards, etc.—have their own dedicated spaces separate from low-risk work. Many also include freezer farms, or rooms with augmented cooling to handle equipment like -80° freezers. It's also important to keep lab spaces separate from eating and office areas.

Anderson reports there's also a push toward open lab spaces to encourage interdisciplinary interactions and collaborative research; this approach is perfectly acceptable, as long as the containment of hazards comes before layout preferences.

- **Engineering controls.** Engineering controls are those processes and pieces of equipment that prevent the release of contaminants into the workplace. To the extent feasible, the work environment and the job itself should be designated to eliminate hazards or reduce personnel exposure to hazards. Examples of engineering controls include fume hoods, biosafety cabinets, glove boxes, safety showers, and more.

- **Procedures.** Examining procedures requires looking through a big-picture lens. Anderson recommends asking the following questions: Are your current procedures as safe as they could be? Are there better processes to achieve the same result? Don't replicate existing processes and ways of doing things for the sake of history. Is there a better way? A safer way?

- **PPE.** PPE comes back to application, and selecting PPE safely requires asking more design questions. Is specific infrastructure needed to accommodate a particular PPE? If you're using Powered Air Purifying Respirators (PAPRs) in a BSL3 lab, for example, there should be a specific place to store them as well as charge the batteries. Don't keep the status quo for the sake of convenience.

- **People**. For optimal safety, staff must be properly trained. Front line workers should be consulted on lab design and layout, as they're the ones who will be

utilizing the space most often. When it comes to hiring a design team, look for architects with specific laboratory experience. The latter point is especially important, as there are a number of considerations only those familiar with the unique requirements of labs will be able to address. For example, if BSL2 work will be conducted in the new space, there must be negative airflow from the hallway into the lab. The exhaust system, then, must be able to meet that need as well as ensure there are enough air changes in the room to keep occupants safe. In addition, rather than relying on running extention cords and using power strips, lab designers know the importance of ensuring sufficient electrical capacity and outlet options for the different types of equipment the lab will house.

The Importance of Flexibility: Spotlight on Biosafety Cabinets

One of the key considerations for lab managers and safety officers looking into a life science lab design or redesign is flexibility. Building infrastructure that can handle future needs is just as important as satisfying immediate objectives — in fact, it may be even more important if you're taking the long view on both ROI, efficiency, and utility.

There are a number of different types of equipment that can add flexibility to a laboratory design initiative, including water systems, vacuum lines, glassware washers, and Class II biosafety cabinet usage. The latter is the specialty of Brian Garrett, Product Manager and LEED Green Associate for Labconco Corporation.

In his experience working with both design teams and lab teams, Garrett reports there can often be a gap in communication present that can drastically affect the outcome of a project. Garrett recalled specifically reviewing plans for a project for DNA Genotek in which they "had planned the one thing you cannot do with multiple Type B2 biosafety cabinets, and that is to manifold — or plumb — them together, as the systems will have serious issues."

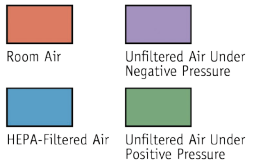
Instead of making this mistake, the lab adopted Class II, Type C1 biosafety cabinets, maintaining the required chemical protection while using the existing manifolded design. The result? A savings of roughly $75,000 in change orders alone.

Another instance where flexibility saved the day was at Creighton University in Omaha, Nebraska. They commissioned an energy savings committee to look at projects that would realize an ROI of five years or better for energy payback. The team completed a number of initiatives, including adding control valves for chilled water usage and switching to LED lights. Their biggest endeavor, however, was reexamining their usage of their aging B2 biosafety cabinets. Instead of replacing the units with newer B2s which can often exhaust more than a chemical fume hood, they opted for Type C1 technology instead, keeping the infrastructure they already had while adding the flexibility of a unit that can operate in either Type A or Type B mode (see Figures 1 and 2).

Figure 1

Type A Mode

When operated in Type A mode, room air is drawn into the inlet grille and directed below the plenum of the work surface where it mixes with air that has passed over the areas on either side of the Chem-Zone. This contaminates air mixture is drawn through the back plenum where it is recirculated through the supply HEPA filter and back over the work area. Air passing over the Chem-Zone is directed through the exhaust HEPA filter and returned to the room.



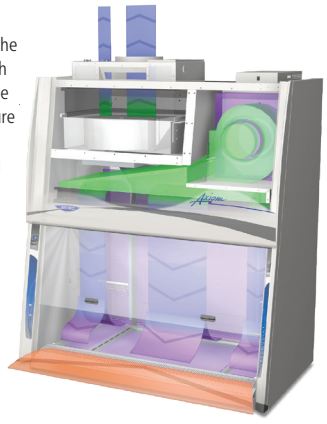
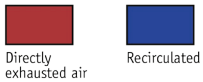
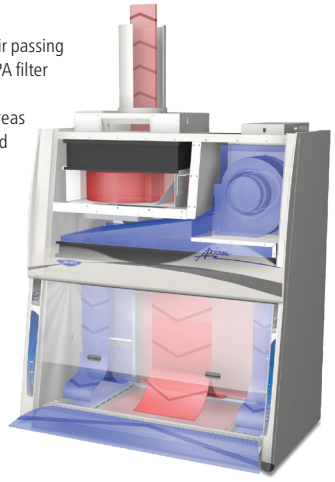


Figure 2

Type B Mode

When opened in Type B mode, 100% of the air passing over the Chem-Zone through the exhaust HEPA filter and delivered to the ductwork where house exhaust can take over. Air passing over the areas on either side of the Chem-Zone is recirculated through the cabinet.





Protecting your laboratory environment
LABCONCO

Figure 3

15 Year Costs	Type A2	Type A2 w/ canopy	Type B1	Type B2	Type C1 in A mode	Type C1 in B mode
Upfront installation	$300	$400	$5,150	$5,150	$300	$400
Lifetime maintenance	$4,500	$4,500	$4,500	$4,500	$4,500	$4,500
Lifetime operation	N/A	$40,500	$40,500	$87,000	N/A	$42,000
Estimated total cost	$4,800	$45,400	$50,150	$96,650	$4,800	$46,900

Upfront installation costs include labor, ductwork, blower (where applicable) and electrical hook up.
Lifetime maintenance costs include HEPA filters and annual certification.
Lifetime operation cost is cost of exhausted air of $8/year/CFM for 15 years.

The result? Creighton realized an ROI of 2.6 years—just over half of the minimum requirement for the project group—and saw an energy savings of over $52,000. Garrett reports those figures are almost double now, as the university added six more Type C1 biosafety cabinets since the case study data was calculated. For a breakdown of savings from a Type C1 compared to Type A and Type B biosafety cabinets, see Figure 3.

In sum, it's clear proper communication and a holistic approach to the laboratory design process can lead to safer, more efficient spaces that cost less to both build and operate — a win-win.

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Puromycin Dihydrochloride	58-58-2	500 mg	75844-854	Each
SB 431542	301836-41	10 mg	75844-602	Each
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