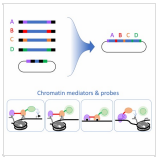

Jan 19, 2021

Rapid Single-Pot Assembly of Modular Chromatin Proteins for Epigenetic Engineering

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ABSTRACT

Chromatin is the nucleo-protein complex that organizes genomic DNA in the nuclei of eukaryotic cells. Transcriptional regulators and enzymes are key mediators of chromatin states that affect gene expression and consequently cell phenotype. Many natural chromatin mediators contain sub-domains that can be isolated and recombined to build novel regulators and live cell probes. A wide variety of synthetic chromatin proteins can be constructed from the diverse array of known natural chromatin proteins and synthetic variants. The process of engineering chromatin mediators and probes produces new tools for cell engineering and deepens our understanding of the mechanism by which chromatin features, such as modifications of histones and DNA, contribute to the epigenetic states that govern DNA-templated processes. To support efficient exploration of the large combinatorial design space of synthetic chromatin proteins, we have developed a Golden Gate assembly method for one-step construction of recombinant protein-encoding DNA. A set of standard 2-amino acid linkers allow facile assembly of any combination of up to four protein modules, obviating the need to design different compatible overhangs to ligate different modules. Beginning with the identification of protein modules of interest, a synthetic chromatin protein can be built and expressed *in vitro* or in cells in under two weeks.

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Golden Gate, molecular cloning, chromatin, epigenetic engineering

IMAGE ATTRIBUTION

All images were created by the authors of this protocol.

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GUIDELINES

Chromatin epigenetic engineering is an emerging area of cellular engineering that aims to control gene expression states by reconstructing chromatin, the system of proteins, RNA, and DNA that organizes the genome within eukaryotic nuclei (**Fig. 1a**) and archaebacteria. Chromatin has captured the interest of cell engineers because of its key role in controlling the timing, magnitude, and persistence of gene activation and silencing [1-3], which affects cell and tissue behavior in animals, plants, and fungi. Chemical inhibition of chromatin-modifying enzymes is an important technique that has helped to establish and advance chromatin epigenetic engineering. The emergence of epigenetic therapy for cancer in the early 1990's and its subsequent development [2,4] have demonstrated that inhibiting chromatin-modifying enzymes alters global chromatin states, changes the expression of dozens to hundreds of genes, and alters cell behavior by, for instance, inducing apoptosis, senescence, and immune signaling. The differentiation of healthy cells can also be guided by chromatin enzyme inhibitors [5]. Treatment of human cells with chromatin enzyme inhibitors induces global changes in chromatin features, for instance loss of H3K27me3 [6], gain of histone acetylation [7], and loss of DNA methylation [8]. Small compounds are powerful tools for regulating gene expression through chromatin, but they can affect abundant non-chromatin proteins outside of the nucleus (e.g. tubulin) [9] rely on the presence of drug-sensitive target enzymes, and lack site specificity in the genome [10]. To overcome these limitations, engineered chromatin proteins can be targeted to the nucleus and to specific genomic loci.

Much work in chromatin epigenetic engineering has focused on two key mediators of chromatin structure and function: chromatin-modifying enzymes and transcriptional regulators. Many of these proteins contain subdomains that maintain their intrinsic activity after they are decoupled from other domains in the endogenous protein [11]. The chromatin-modifying enzyme EP300 (Uniprot Q09472) interacts withmodified histones through its bromodomain (BRD) and plant homeodomain (PHD) regions, and acetylates histone lysine residues through its histone acetyltransferase (HAT) domain [12] (**Fig. 1a**) Transcriptional regulators, also known as reader-effectors, interact with modified histones and regulate chromatin remodeling and transcriptional initiation. BPTF (Bromodomain PHD Finger Transcription Factor, Uniprot Q12830) contains histone-binding domains at its C-terminus and a N-terminal domain that interacts with the nucleosome remodeling complex NURF (**Fig. 1a**).

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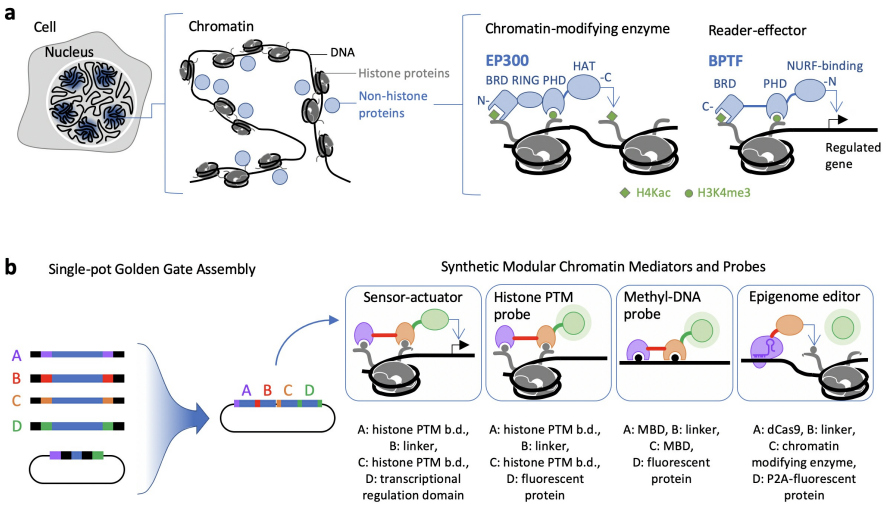

Figure 1 | Chromatin mediator structure and design. (a) Chromatin is a system of nuclear proteins (histones and non-histone proteins) and nucleic acids that organize and regulate genomic DNA. Chromatin enzymes generate or erase modifications on histones or DNA. EP300 interacts with histone modifications (H4ac and H3K4me3) and acetylates histone tails [12]. Reader-effector proteins such as BPTF interact with modified histones and regulate gene expression. (b) Our protocol describes a procedure to assemble modular protein domains into four-part fusion proteins that bind chromatin features and regulate genes (reader-actuators), fluorescently label chromatin enrichment in live cells (histone PTM probes and methyl-DNA probes), and alter chromatin features at specific sites (epigenome editors).

Synthetic proteins that interact with the epigenome

Reader-effector proteins are generally composed of two functional modules. One module is a reader domain that binds a specific chromatin feature such as a histone post-translational modification or 5-methyl-cytosine at CpG dinucleotides (5meCpG). The second module is a transcriptional regulator that can either enhance or inhibit RNA polymerase activity (transcription) at genes. Here, we refer to readers as “sensors” and effectors as “actuators.” (**Fig. 1b**) Synthetic sensor-actuators regulate (activate or repress) a set of genes by interacting with chromatin modifications that are shared across the gene group. For instance, we linked a H3K27me3-binding domain with the VP64 transcriptional activation domain to co-activate repressed genes in cancer cells [13, 14]. A similar broad effect on gene expression can be achieved with FDA-approved chromatin enzyme inhibitors (e.g. AZA, DAC, HDAC inhibitors), but sensor-actuator proteins can be targeted to the nucleus via a nuclear localization signal (NLS) and recruit endogenous transcriptional regulators to their target sites, enabling programmable gene control. In addition to VP64, dozens of transcriptional activator and repressor modules can be used as actuators [15-18]. Chromatin sensors can also be linked to fluorescent proteins and used as probes to locate sub-nuclear regions that are enriched for specific chromatin features as cells respond to stimuli and perturbations, or undergo development. Such probes have been used to detect histone PTMs [19-22] and DNA methylation [23].

To date, five general classes of naturally-occuring eukaryotic chromatin sensor domains have been experimentally validated as composable modules [11, 24]: plant homeodomain Cys4-His-Cys3 (PHD), chromodomain (CD), bromodomain (BRD), baculovirus inhibitor of apoptosis repeat (BIR), and methyl-DNA binding domain (MBD). Each domain recognizes a distinct biochemical feature within chromatin: PHD, histone H3K4me3; CD, histone H3K27me3 or H3K9me3; BRD, histone H3/H4 lysine acetylation; BIR, histone H3T3p; MBD, 5meCpG. Genetic studies [25] and emerging technologies to interrogate libraries of histone binding domains and histone PTMs [26-30] continue to reveal diverse natural sensors. The recent discovery of a synthetic mutant H3K9me3-binding CBX1 protein [31] suggests it is possible to artificially expand the diversity of sensors. Here, we present a general DNA assembly method to rapidly assemble and test any four-part combination of sensors and actuators or fluorescent tags.

Synthetic proteins that edit the epigenome

Epigenome editing allows precise control of DNA methylation and histone modifications at specific loci such as genes and non-coding enhancer elements. This method can be performed with fusion enzymes that include a DNA-binding module, such as zinc finger (ZF) proteins, Transcription Activator-Like Effector (TALE) proteins, or dead Cas9-gRNA complexes (dCas9) to target a chromatin-modifying enzyme to a single DNA sequence [11]. Such fusion proteins have been used to generate or erase DNA methylation or histone modifications at specific loci in immortalized human cell lines [32-34]. These modifications lead to gene activation or repression, depending upon the type of chromatin feature that is generated at the target locus. To aid the use and development of the large

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array of chromatin-modifying enzymes that have been described elsewhere [33, 36] we have developed a single-pot assembly platform to build epigenome editors that contain the popular dCas9 targeting module [18, 34, 37-43] and up to three additional protein-coding regions such as protein linkers, chromatin modifying catalytic domains, and fluorescent tags.

Experimental design

The workflow for assembling and validating synthetic chromatin protein-coding DNA in one of the intermediate cloning vectors or expression vectors described herein can be completed in about seven days (**Fig. 2**). Cell-free expression of fusion proteins from vector GGDestX1-Amp can be completed in one day. Downstream applications are diverse and application-dependent. Therefore, we refer to protocols and examples published elsewhere in the sections "Testing sensor-actuators, histone PTM probes, and methyl-DNA probes," and "Testing epigenome editor activity in cells with GGDestX2-Amp." Histone-binding specificity of sensors can be examined *in vitro* using small quantities of cell-free expressed proteins in two days. Fusion protein activity in living cells may require one week or more depending upon the target cells, gene expression assays, and chromatin profiling assays that are used.

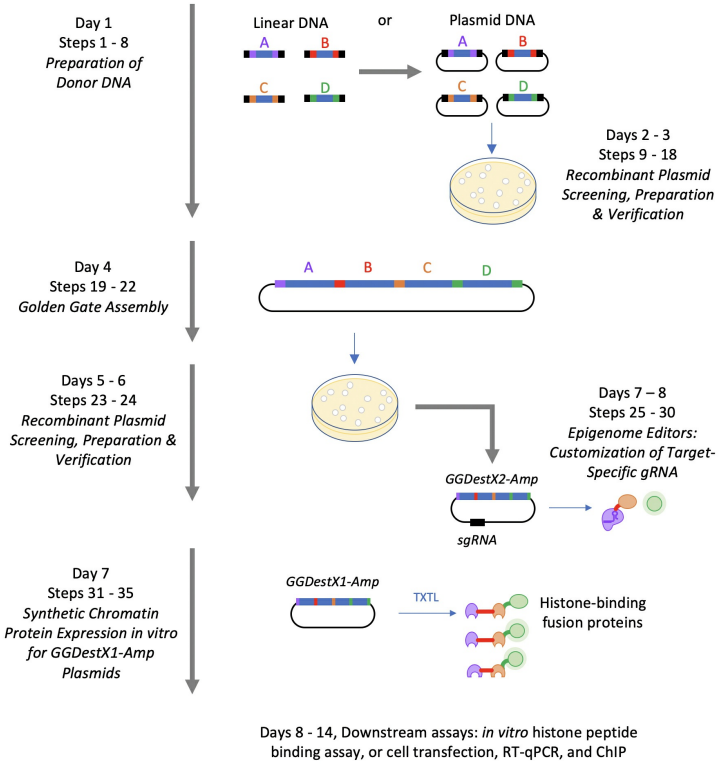

Figure 2 | Timeline and overview of experiments.

Design of sensor-actuators, histone PTM probes, and methyl-DNA probes.These synthetic chromatin proteins contain a combination of up to four parts (e.g. sensor, actuator, fluorescent tag, signal peptide, etc.) each at positions A, B, C, D in the final recombinant DNA product (**Fig. 1b**). These proteins should contain at least two sensor modules for strong binding with chromatin features; tandemly linked chromatin-binding domains enhance the overall avidity for the chromatin target [44, 45]. The fusion protein should also include a fluorescent protein tag to support real-time measurement of expression in vitro and in cells. Positions A and C can be tandem histone or methyl-DNA binding domains. Position B can be a linker of any length or structure. The effect of the linker on fusion protein behavior should be empirically tested. We observed that linker types affect the binding of tandem PCDs with H3K27me3 *in vitro* [45]. Selection of the actuator module(s). An actuator module should be selected based on the desired regulatory effect at anticipated target genes, as discussed in a previous review (see Tekel and Haynes, Figure 3 [11]).

Design of epigenome editors. Epigenome editors are composed of at least three parts: catalytically inactive dCas9, a linker protein, and a chromatin-modifying enzyme or transcriptional regulator. The fourth position can be a fused or cleaved fluorescent protein tag (e.g. the P2A signal followed by a fluorescent protein, **Fig. 1b**). A target site-specific guide RNA (gRNA) must be co-expressed with the dCas9 fusion protein. We have built an expression

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vector for this purpose and describe its use in this protocol.

Approaches for module (donor) construction

Single-pot Golden Gate assembly reactions (illustrated in **Fig. 1b, 2**) require two types of DNA inputs: donor and destination molecules. In the Golden Gate scheme described here, each donor (modules A, B, C, and D) includes an open reading frame (ORF) flanked by a unique 4 or 6 bp spacer sequence and a recognition site for the Type IIS restriction enzyme BbsI (**Fig. 3**). Cleavage with BbsI generates 4 bp 5′ overhangs at each end and removes the BbsI binding sites. Each complementary pair of 4 bp overhangs dictates the order in which each ORF will appear in the final assembly: A followed by B, then C, and then D. Bases are included in the internal 6 bp spacers to avoid frameshift mutations that would otherwise be created by 4 bp spacers in the final assembly. Assemblies can be reduced from four to three, two, or one fragment by generating hybrids that start and end with different overhangs. For instance, A and B can be reduced to a single A-B hybrid by placing the sticky end for A on the left (5′- side) and the sticky end for B on the right (3′- side) of the donor DNA fragment.

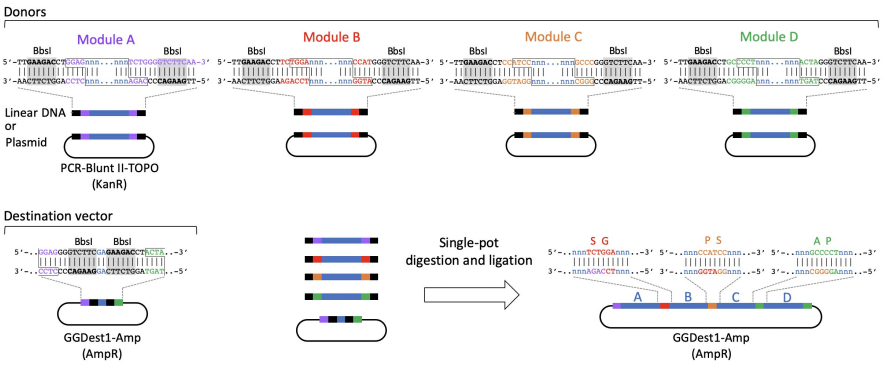

Figure 3 | Schematic of Golden Gate assembly of epigenetic regulators composed of up to four modular parts. The first and last codon (nnn) of each module is positioned next to a 4-bp overhang that guides directed assembly of the modules (A, B, C, and D). Single-pot digestion and ligation with Type IIS enzyme BbsI and T4 DNA ligase results in four modules linked in frame by amino acids with small R-groups: serine-glycine (S G), proline-serine (P S), and alanine-proline (A P).

Linear DNA donors. PCR is used to add BbsI sites and 4-bp 5′ overhangs to each end of the modules for the assembly. Linear PCR amplicons can be used without cloning them as plasmids. One key advantage of this approach is that linear fragment donors lack an antibiotic resistance gene and will not produce false-positive clones on the Golden Gate colony plate.

Plasmid DNA donors. Donor plasmids are generated using Zero Blunt™ TOPO™ PCR cloning. This method introduces the blunt-end PCR products into the vector PCR-Blunt II-TOPO. This vector is commercially provided as a linear dsDNA fragment with Topoisomerase I covalently linked to the 3′ phosphates on each end of the vector. The PCR-Blunt II-TOPO vector contains a kanamycin resistance gene for selection of successfully transformed cells. Proper ligation of the PCR product into PCR-Blunt II-TOPO disrupts the lacZα-ccdB gene, providing another selection marker to ensure the growth of positive recombinants. Plasmid donors are recommended for long-term storage and easy error-free regeneration of frequently-used donor parts (via bacterial cloning). Successful Golden Gate assembly is possible with both plasmids and with linear fragments (**Fig. 4**).

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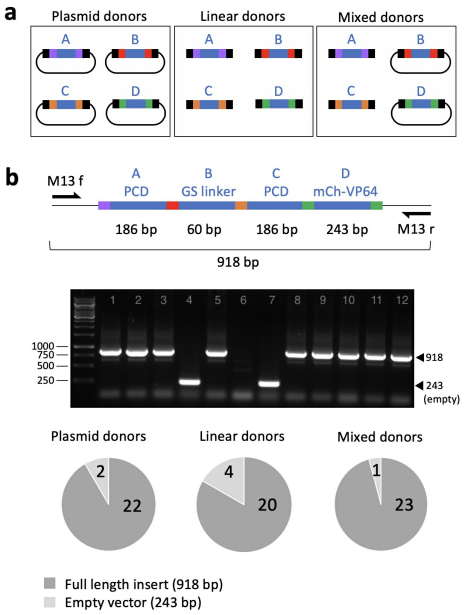

Figure 4 | Golden Gate assembly in the destination vector GGDest1-Amp using combinations of plasmid and linear donors. Donors were generated with primers synthesized by Integrated DNA Technologies (custom oligos, IDT.com). (a) Map of the desired assembly and schematic of Golden Gate reactions and colony PCR. (b) For each assembly (plasmid, mixed, or linear), 24 colonies were picked for colony PCR (half arrows = primers). A representative DNA electrophoresis gel is shown. Pie charts show frequencies of amplicons from successful assemblies (918 bp) and empty GGDest1-Amp vectors (243 bp).

Destination vectors

The destination vector provides the plasmid backbone that carries the fragments assembled from the donor parts in the Golden Gate reaction (e.g. GGDest1-Amp, used for the reaction illustrated in **Fig. 3**). Scientists who wish to use or design their own destination vector should adhere to the following requirements for Golden Gate compatibility: (1) absence of the donor Type IIS (BbsI) sites except where the assembled insert will be ligated, and (2) an antibiotic resistance gene (e.g. AmpR) that is different from the donors' resistance gene (e.g. KanR) if plasmid donors are being used.

Shuttle destination vector. A shuttle destination vector is an intermediate cloning vector that carries a BbsI insert site flanked by other useful features such as additional cloning sites and expression regulators. The ORF is then excised via restriction digest from the shuttle vector and cloned into other vectors for specific downstream applications (e.g. bacterial or mammalian expression). Shuttle vectors are recommended when it is not easy or possible to eliminate BbsI sites from the final expression plasmid. We have designed three shuttle destination vectors, GGDest1-Amp, GGDest2-Amp, and GGDest3-Amp, that flank the Golden Gate construct with different features so that the inserts can be transferred into expression vectors (**Fig. 5**). GGDest1-Amp supports transfer into mammalian expression vector pcDNA3.1+ (Invitrogen). GGDest2-Amp and GGDest3-Amp enable transfer into pET28 and pTXB1 (respectively) for bacterial expression and high-yield purification of recombinant proteins for in vitro histone peptide binding assays [45] or other experiments.

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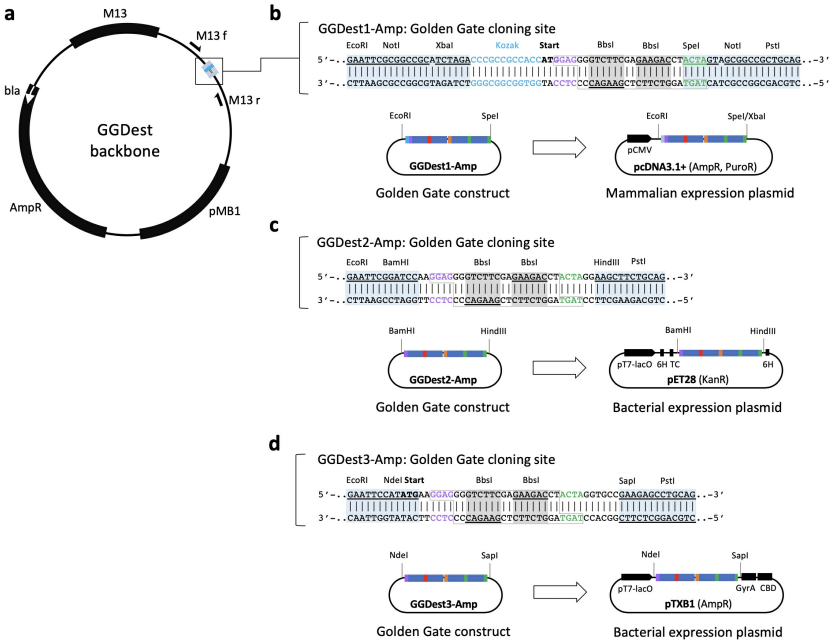

Figure 5 | Maps of Golden Gate shuttle destination vectors. (a) The backbone includes an ampicillin selection marker (AmpR), a high-copy number origin of replication (pMB1), and is derived from vector V0120 (C. Ajo-Franklin, unpublished). (b) GGDest1-Amp is used for transfer of Golden Gate (GG) assembled constructs into BioBrick compatible [46] vectors such as pcDNA3.1+ (Invitrogen) for mammalian expression. GG assembly at the BbsI sites places BioBrick cloning sites and a Kozak sequence upstream and BioBrick cloning sites downstream of the insert. (c) GG assembly in GGDest2-Amp flanks the construct with BamHI and HindIII sites so that it can be cloned in-frame with 6-histidine (6-his) and thrombin cleavage (TC) tags in the pET28 bacterial expression vector. (d) GG assembly in GGDest3-Amp flanks the construct with NdeI and SapI so that it can be cloned in pTXB1 (New England Biolabs) in-frame with the Mxe GyrA intein and a chitin-binding domain (CBD).

Expression destination vector. Fragments can be assembled in the expression vector itself as long as the plasmid sequence is compatible with Type IIS (BbsI) assembly, i.e. it must not contain any BbsI sites. We have designed a vector called GGDestX1-Amp for cell-free transcription and translation in a bacteria-derived lysate (MyTXTL, Arbor Biosciences) (**Fig. 6a, b**). Another vector called GGDestX2-Amp enables the expression of GG-assembled epignome editors along with a customized guide RNA (**Fig. 6c**).

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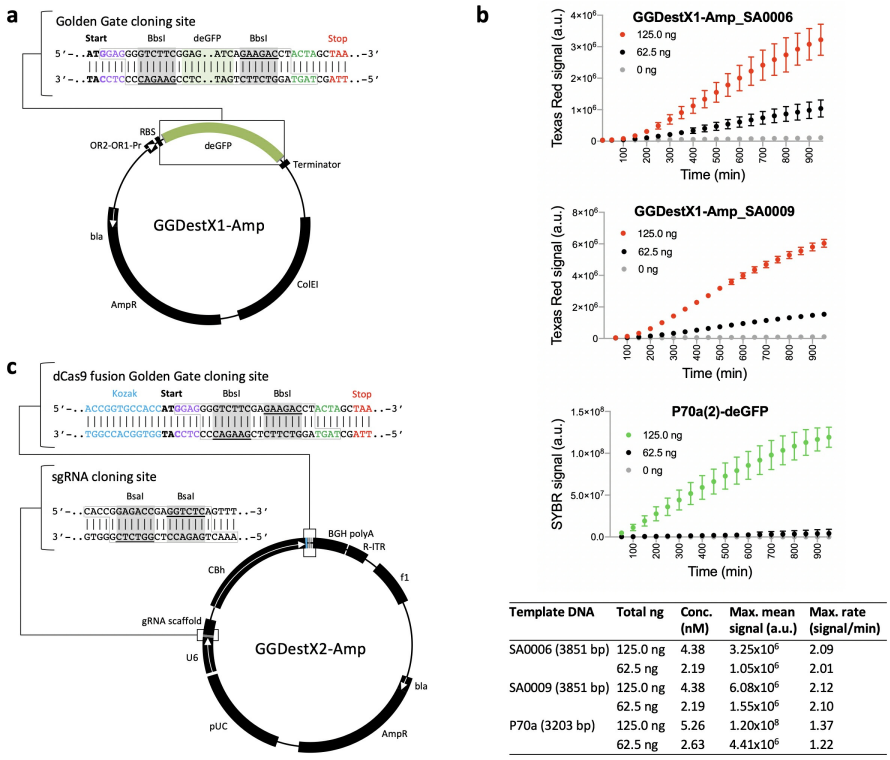

Template DNA	Total ng	Conc. (nM)	Max. mean signal (a.u.)	Max. rate (signal/min)
SA0006 (3851 bp)	125.0 ng	4.38	$3.25x10^6$	2.09
	62.5 ng	2.19	$1.05x10^6$	2.01
SA0009 (3851 bp)	125.0 ng	4.38	$6.08x10^6$	2.12
	62.5 ng	2.19	$1.55x10^6$	2.10
P70a (3203 bp)	125.0 ng	5.26	$1.20x10^8$	1.37
	62.5 ng	2.63	$4.41x10^6$	1.22

Figure 6 | Maps of Golden Gate expression destination vectors. (a) GGDestX1-Amp, a modified version of P70a(2)-deGFP (Arbor Biosciences), is used for cell-free expression of synthetic chromatin fusion proteins. (b) Cell-free expression of mCherry-tagged sensor-actuators (SA0006 and SA0009) cloned in GGDestX1-Amp or EGFP from the P70a plasmid (control). The sensor-actuators have the same structure as the construct shown in **Fig. 4**. Each point is the mean of triplicate reactions. Bars = standard deviation. (c) GGDestX2-Amp is a modified version of pX330 (Addgene plasmid #42230) [47].

Testing sensor-actuators, histone PTM probes, and methyl-DNA probes

Prior to their use in living cells, engineered chromatin-binding proteins should be validated for target specificity in vitro. We recommend GGDestX1-Amp for this purpose. It is useful to benchmark the synthetic chromatin protein design process by first determining the expression efficiency of protein-encoding plasmids (**Fig. 6b**). Here, we describe an efficient procedure for cell-free expression and detection of proteins in a real time thermocycler. Next, the on-target and off-target binding affinity (apparent Kd) of cell-free expressed products can be determined for histone PTM-binding proteins using an ELISA-style assay with modified histone peptides as we have described previously [48]. Other in vitro assays can be used to determine the affinity of MBD-containing proteins for methylated DNA, such as commercial ELISA-based kits and methyl-DNA capture columns [24]. Once on-target specificity of the engineered chromatin-binding protein is determined in vitro, it should be reassembled in a shuttle destination vector (e.g. GGDest1-Amp, **Fig. 5b**) and then cloned into an appropriate cellular expression vector (e.g. pcDNA3.1+ for mammalian cells, **Fig. 5b**).

Testing epigenome editor activity in cells with GGDestX2-Amp

To test the function of an epigenome editor a useful genomic target site in a specific cell line or organism should first be identified. The target site's baseline chromatin state should be determined via chromatin immunoprecipitation (ChIP) in advance so that changes in chromatin features of interest can be reliably measured. We strongly recommend testing several gRNA target sites; gRNA/dCas9 binding and/or regulator function can be position-dependent [18, 34, 49, 50]. Changes in the expression of endogenous gene targets should be measured via reverse-transcription PCR, at minimum. If possible, a synthetic target locus that expresses a fluorescent protein can be used to determine changes in gene expression states in real time [51].

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MATERIALS TEXT

REAGENTS

Donor DNA preparation

- PCR primers for donor fragments (see **Table 1**)
- ☒ Molecular grade H20 **Sigma**
- **Aldrich Catalog #W4502**
- ☒ Q5 High-Fidelity PCR Kit - 200 rxns **New England**
- **Biolabs Catalog #E0555L**
- ☒ GenElute™ PCR Clean-Up Kit **Sigma**
- **Aldrich Catalog #NA1020**
- ☒ Zero Blunt™ TOPO™ PCR Cloning Kit without competent cells **Thermo Fisher**
- **Scientific Catalog #450245**
- ☒ NEB Turbo Competent E.coli (High Efficiency) - 6x0.2 ml **New England**
- **Biolabs Catalog #C2984I**
- ☒ SOC Outgrowth Medium - 100 ml **New England**
- **Biolabs Catalog #B9020S**
- ☒ LB Broth with agar (Lennox) **Sigma**
- **Aldrich Catalog #L7533**
- ☒ Kanamycin sulfate **Sigma**
- **Aldrich Catalog #60615**

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GenElute™ HP Plasmid Miniprep Kit **Sigma**

- **Aldrich Catalog #NA0160**

Colony PCR screening

DreamTaq Green PCR Master Mix (2X) **Thermo**

- **Fisher Catalog #K1081**
- PCR primers (see **Table 2**)
- Reagents for DNA electrophoresis:

TAE (Tris Acetate-EDTA) Buffer **Contributed by**

- **users Catalog #T6025**

Agarose **Sigma**

- **Aldrich Catalog #A9539**

SYBR™ Safe DNA Gel Stain **Thermo Fisher**

- **Scientific Catalog #S33102**

GeneRuler 1 kb DNA Ladder, ready-to-use **Thermo**

- **Fisher Catalog #SM0314**

TriTrack DNA Loading Dye (6X) **Thermo**

- **Fisher Catalog #R1161**

Recombinant DNA purification and analysis

GenElute™ HP Plasmid Miniprep Kit **Sigma**

- **Aldrich Catalog #NA0160**

FastDigest Green Buffer (10X) **Thermo Fisher**

- **Scientific Catalog #B72**

FastDigest EcoRI **Thermo Fisher**

- **Scientific Catalog #FD0274**

FastDigest BcuI (SpeI) **Thermo Fisher**

- **Scientific Catalog #FD1254**

FastDigest BamHI **Thermo Fisher**

- **Scientific Catalog #FD0055**

FastDigest HindIII **Thermo Fisher**

- **Scientific Catalog #FD0504**

FastDigest NdeI **Thermo Fisher**

- **Scientific Catalog #FD0583**

FastDigest LguI (IIs class) (SapI) **Thermo Fisher**

- **Scientific Catalog #FD1934**

FastDigest XhoI **Thermo Fisher**

- **Scientific Catalog #FD0694**

FastDigest XbaI **Thermo Fisher**

- **Scientific Catalog #FD0684**
- Reagents for DNA electrophoresis (see “Colony PCR screening”)

Golden Gate assembly

- Destination vector (choose one of the following, see **Fig. 5 and Fig. 6**): GGDest1-Amp (Addgene, 157649), GGDest2-Amp (Addgene, 157650), GGDest3-Amp (Addgene, 157651), GGDestX1-Amp (Addgene, 157652), or GGDestX2-Amp (Addgene, 157654)

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T4 DNA Ligase Reaction Buffer - 6.0 ml **New England**

- **Biolabs Catalog #B0202S**

Quick Ligation Kit - 150 rxns **New England**

- **Biolabs Catalog #M2200L**

FastDigest BpiI **Thermo**

- **Fisher Catalog #FD1014** (BbsI)

LB Broth with agar (Lennox) **Sigma**

- **Aldrich Catalog #L7533**

Ampicillin sodium salt **Sigma**

- **Aldrich Catalog #A9518**

Type IIS "drop-in" cloning of gRNA target sequence for GGDestX2-Amp

- GGDestX2-Amp with a dCas9 fusion ORF assembled at the BbsI site
- ssOligos to generate dsOligos (see **Table 4**)

T4 DNA Ligase Reaction Buffer - 6.0 ml **New England**

- **Biolabs Catalog #B0202S**

T4 Polynucleotide Kinase - 500 units **New England**

- **Biolabs Catalog #M0201S**

Buffer G (10X) **Thermo**

- **Fisher Catalog #BG5**

DTT, 100mM

- (Dithiothreitol) **Promega Catalog #P1171**

Adenosine-5 Triphosphate (ATP) - 1 ml **New England**

- **Biolabs Catalog #P0756S**

Eco31I (BsaI) (10 U/µL) **Thermo**

- **Fisher Catalog #ER0291**

T7 DNA Ligase - 750,000 units **New England**

- **Biolabs Catalog #M0318L**

Plasmid-Safe™ ATP-Dependent DNase and 10X Reaction

- Buffer **Lucigen Catalog #E3101K**
- Primer for Sanger sequencing (see **Table 2**)

Cell free expression from GGDestX1-Amp plasmids

- GGDestX1-Amp with fusion protein assembled at the BbsI site (≥ 40 ng/µL)
- GGDestX1-Amp vector (control)

pTXTL-P70a(2)-deGFP HP Sigma70MM kit **Arbor**

- **Biosciences Catalog #502138**

myTXTL Sigma 70 Master Mix Kit **Arbor**

- **Biosciences Catalog #507024**

Bradford Reagent **Sigma**

- **Aldrich Catalog #B6916**

Pierce™ Bovine Serum Albumin Standard 2 mg/mL **Thermo Fisher**

- **Scientific Catalog #23210**

EQUIPMENT AND CONSUMABLES

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- Tips for Rainin LTS 1000 µL (1200 µL max) **pipette.com Catalog #LE-1000**
- Tips for Rainin LTS 200 µL **pipette.com Catalog #LE-250**
- Tips for Rainin LTS 20 µL **pipette.com Catalog #LE-20**
- CAPP Harmony Serological Pipette Variety Pack (5mL 10mL and 25mL) **pipette.com Catalog #SP-VAR1**
- Microcentrifuge Tubes: 0.6 mL **Fisher Scientific Catalog #05-408-120**
- Microcentrifuge Tubes: 1.5 mL **Fisher Scientific Catalog #05-408-129**
- Microcentrifuge Tubes: 2.0 mL **Fisher Scientific Catalog #05-408-138**
- MicroAmp™ 8-Tube Strip with Attached Domed Caps 0.2 mL **Thermo Fisher Scientific Catalog #A30589**
- Corning™ 100 x 15 mm Polystyrene Petri Dishes **Fisher Scientific Catalog #07-202-011**
- MilliporeSigma™ Novagen™ ColiRollers™ Plating Beads **Fisher Scientific Catalog #71-013-4**
- ProFlex 3x32 well PCR System (Fisher Scientific Catalog #44-840-73)

ProFlex™ PCR System, 3 x 32-well thermocycler

Applied Biosystems **44-840-73**

- Shaking Incubator - MaxQ 4450 (Thermo Fisher Scientific Catalog #SHKE4450)

MaxQ™ 4450 Benchtop Orbital Shaker shaking incubator

Thermo Scientific **SHKE4450**

Small Incubated Benchtop Orbital Shaker; Electrical: 120V 50/60Hz; Temperature: Ambient+5° to 60°C; Speed: 5 to 500 ±1 rpm

- iBright FL1000 gel and blot imager (Thermo Fisher Scientific Catalog #A32752)

iBright™ FL1000 Imaging System imager

Invitrogen **A32752**

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- Compact Dry Baths/Block Heaters, Model D (VWR Catalog #0753-608)

Compact Dry Bath/Block Heater, Model D
dry bath
Thermo Scientific **10753-608**
Electrical: 120V; Dimensions: 200L x 200W x 25H mm (7.9 x 7.9 x 1"); Weight: 1.5 kg (3.3 lbs.) without Blocks

PYREX® Erlenmeyer Flasks Graduated Wide Mouth (250 mL) **VWR**

- **International Catalog #29140-045**

Nunc™ 14 mL Round-Bottom Tube **Fisher**

- **Scientific Catalog #12-565-971**
- Horizontal gel electrophoresis system, e.g. Owl EasyCast B2 Mini Gel System (Fisher Scientific Catalog #09-528-110B)

Owl™ EasyCast™ B2 Mini Gel Electrophoresis Systems
electrophoresis system
Thermo Scientific **09-528-110B**

- PowerPac Universal Power Supply (Bio-Rad Catalog #1645070)

PowerPac Universal Power Supply
power supply
Bio-Rad **1645070**

- NanoDrop One Spectrophotometer (Thermo Fisher Scientific Catalog #ND-ONE-W)

NanoDrop™ One UV-Vis Spectrophotometer
spectrophotometer
Thermo Scientific **ND-ONE-W**
Sample Volume (Metric): Minimum 1µL; Spectral Bandwidth: ≤1.8 nm (FWHM at Hg 254 nm); System Requirements: Windows™ 8.1 and 10, 64 bit; Voltage: 12 V (DC); Wavelength Range: 190–850 nm

- Benchtop microcentrifuge, e.g. Labnet Spectrafuge 24D (VWR International Catalog #490008-862)

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Labnet Spectrafuge™ 24D Microcentrifuge
centrifuge

Labnet **490008-862**

Electrical: 120/230V, 50/60 Hz; Dimensions: 23.5 x 29.3 x 21.6 cm (9.25 x 11.5 x 8.5"); Max. capacity: 24 x 1.5/2.0 mL; Max. rcf: 16,300 x g; Max. speed: 13,300 rpm; Temp. range: 4°C to 35°C

- QuantStudio 6 real-time thermocycler (Thermo Fisher Scientific Catalog #4485689)

QuantStudio™ 6 Flex Real-Time PCR System, 96-well
thermocycler

Applied Biosystems **4485689**

Bemis™ Parafilm™ M Laboratory Wrapping Film **Fisher**

- **Scientific Catalog #13-374-12**
-

MicroAmp™ EnduraPlate™ Optical 96-Well Clear Reaction Plates with Barcode **Thermo Fisher Scientific Catalog #4483352**

MicroAmp™ Clear Adhesive Film **Thermo Fisher**

- **Scientific Catalog #4306311**
- BioTek Cytation 1 96-well plate reader (Fisher Scientific Catalog #BTCYT1FAV)

BioTek™ Cytation™ 1 Cell Imaging Multi-Mode Reader
plate reader

BioTek **BTCYT1FAV**

Application: Fluorescence and brightfield imaging, filter based fluorescence and luminescence, monochromator based UV Vis absorbance

Greiner Bio-One 96-Well Non-treated Polypropylene Microplates **Fisher**

- **Scientific Catalog #07-000-113**

REAGENT SETUP

- TAE electrophoresis solution: Dilute TAE buffer solution in distilled water to 1X, and keep at room temperature for up to 6 months.
- Agar plates: Dissolve **17.5 g** LB broth with agar in **500 mL** distilled water and autoclave on a short liquid cycle (**00:20:00** sterilization, no drying) to fully melt the agar. Let the buffer cool to **60 °C** before adding the appropriate antibiotic. Pour **10 mL** of LB broth with agar plus antibiotic per plate and let the agar cool overnight

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before storing the plates at 4 °C stacked and inverted (lid-side-down).

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SAFETY WARNINGS

Personal Protective Equipment (PPE): At minimum, a standard cotton/ polyester lab coat and disposable nitrile (or similar) gloves. **Biohazard**: Treat all cell culture liquid waste with bleach (10% final concentration) and dispose of the treated waste according to your institute's environmental health and safety (EH&S) protocol. Discard all disposable items (e.g. micropipette tips, culture tubes, etc.) that have come into contact with growth medium and/or cells as dry biohazard waste. **Recombinant DNA**: The plasmid vectors described in this protocol are not known to support transmission between cells or organisms or high rates of horizontal gene transfer in the environment. **Chemical Warning**: Do not mix ethanol waste with bleach waste. Doing so will produce toxic chloroform vapors.

DISCLAIMER:

The development of this protocol was supported by the NIH NCI (R21CA232244 to K. Haynes).

Preparation of Donor DNA Fragments 3h 20m

1 Generation of linear donor DNA via Q5 HiFi PCR amplification. 35m

1.1 ⚠ 5m

Obtain template DNA for the module(s) of interest that contains a complete open reading frame (ORF) without stop codons.

Ensure that the sequence does not contain any BbsI recognition sites (5'-GAAGAC). If the sequence contains these sites, modify the sequence by creating a silent substitution mutation so that the affected codon corresponds to the same amino acid as the original sequence. This can be achieved with site directed mutagenesis of plasmid DNA that carries the ORF, or after cloning the fragment into the TOPO vector (Step 7), or by purchasing a de novo synthesized fragment with the desired sequence.

1.2 🖵 30m

Designing PCR primers to add BbsI sites to donor DNA. Design forward and reverse primers that include the appropriate restriction site and overhang sequences (see **Table 1**) followed by at least 15 bp (shown as 5'-NNN… in **Table 1**) that bind the template. Synthesize the primers as single-stranded oligos or purchase the primers from a commercial vendor.

The template-binding region of the primer should have a Tm value of 🌡 **65 °C** . Use the NEB Tm Calculator (set for Q5 High-Fidelity DNA Polymerase) and adjust the binding region as needed to achieve this Tm target value (https://tmcalculator.neb.com/#!/main).

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A	B	C
Primer	**Sequence (5'-3')**	**Purpose**
A-Module f	TTGAAGACCTGGAGNNNNNNNNNNNNNNN	Add left BbsI and 4 bp overhang onto the 5' side of module A.
A-Module r	TTGAAGACCCCAGANNNNNNNNNNNNNNN	Add right BbsI and 4 bp overhang onto the 3' side of module A.
B-Module f	TTGAAGACCTTCTGGANNNNNNNNNNNNNNN	Add left BbsI and 4 bp overhang onto the 5' side of module B.
B-Module r	TTGAAGACCCATGGNNNNNNNNNNNNNNN	Add right BbsI and 4 bp overhang onto the 3' side of module B.
C-Module f	TTGAAGACCCATGGNNNNNNNNNNNNNNN	Add left BbsI and 4 bp overhang onto the 5' side of module C.
C-Module r	TTGAAGACCCGGGCNNNNNNNNNNNNNNN	Add right BbsI and 4 bp overhang onto the 3' side of module C.
D-Module f	TTGAAGACCTGCCCCTNNNNNNNNNNNNNNN	Add left BbsI and 4 bp overhang onto the 5' side of module D.
D-Module r	TTGAAGACCCTAGTNNNNNNNNNNNNNNN	Add right BbsI and 4 bp overhang onto the 3' side of module D.

2  10m

Preparation of diluted PCR primers. Prepare working solutions of forward and reverse primers at a final concentration of 10 µM.

3  15m

Q5 HiFi PCR set-up. Set up the following reaction for each donor part as follows.

A	B	C
Reagent	**Vol (µl)**	**Final Concentration**
*Q5 buffer (5X)	5.0	1X
*dNTPs (10 mM)	0.5	0.2 mM
10 µM primer F	1.25	0.5 µM
10 µM primer R	1.25	0.5 µM
DNA	0.5	
*Q5 Polymerase	0.25	
MB H2O	16.25	
Total	25	

*Reagents from the NEB Q5® High-Fidelity PCR Kit.

4  1h

Q5 HiFi PCR. Perform PCR with the following cycle conditions.

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A	B	C	D	E
Cycle Number	**Denature**	**Anneal**	**Extend**	**Hold**
1	98°C, 30 s			
2-26	98°C, 10 s	65°C, 30 s	72°C, 30 s	
27			72°C, 2 min	
Post-reaction				4°C, ∞

5 20m

DNA purification. Purify the linear donor DNA (PCR product) using the GenElute™ PCR Clean-Up Kit according to the manufacturer's guidelines.

6 1h

Gel electrophoresis confirmation of PCR amplified linear donor DNA. Cast a 1% (wt/vol) low EEO agarose gel in 1X TAE buffer with 1:10,000 SYBR Safe DNA Gel Stain. Load **3 µl** of GeneRuler 1 kb DNA ladder in the first well and **3 µl** of purified linear donor DNA with **2 µl** MB H_2O and **1 µl** 6X Tritrack DNA loading dye in the adjacent wells. Run the gel for **00:45:00** at 100V. Successful reactions should yield a single product equal to the size of the donor fragment plus overhangs (**Table 1**).

If you are using linear DNA for Golden Gate assembly, proceed to "Golden Gate Assembly of Fusion Protein Plasmids. If you want to generate donor plasmids, proceed to "Generation of Plasmid Donors" (below).

Generation of Plasmid Donors (optional) 18h 54m

7 15m

Ligation of linear donors into plasmids through Zero Blunt™ TOPO™ PCR Cloning. The PCR-amplified linear donor module, now flanked by the module's specific BbsI sites, is subjected to a Zero Blunt™ TOPO™ PCR Cloning reaction in a PCR tube according to the reaction table below. Reaction time is five minutes at room temperature.

A	B
Reagent	**Volume (µl)**
Fresh PCR product	0.5
*Salt solution	1.0
*pCR-Blunt II-TOPO	1.0
MB H2O	3.5
Total	6.0

*Reagents provided in the Thermo Fisher Scientific Zero Blunt™ TOPO™ PCR Cloning Kit.

8 **Transformation with Zero Blunt™ TOPO™ PCR Cloning Kit ligation products**. 18h 39m

8.1 5m

Pipette the **6 µl** reaction product into a 2.0 mL microcentrifuge tube and add **50 µl** chemically competent DH5α-T cells. Gently tap the tube three times and incubate on ice for **00:05:00** .

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8.2 45s

Heat shock at **42 °C** for **00:00:45** .

8.3 30m

Add **1 mL** SOC outgrowth medium and incubate on a shaking platform at **300 rpm, Room temperature , 00:30:00** .

8.4 3m

Pellet the cells at **16300 x g, Room temperature , 00:03:00** and discard the supernatant.

8.5 18h

Resuspend the pellet(s) in **50 µl** 50 µg/mL kanamycin LB broth. Plate each resuspension onto a pre-warmed 50 µg/mL kanamycin LB agar plate and spread using beads. Incubate at **37 °C** **Overnight** .

Recombinant Plasmid Screening with Colony PCR 20h 17m

9 5m **Plate inspection**. Obtain colony plates from the incubator and inspect for colony growth. Optimal growth for colony PCR will have clearly separated colonies with minimal to no surrounding satellites, allowing precise colony picking with no cross-contamination. Determine the number of colonies to be picked based on the following recommendations:

- Plasmid Donor (Zero Blunt™ TOPO™), 2 - 4 colonies per unique ligation
- Golden Gate ligations, 4 - 6 colonies per unique ligation

10 5m

Preparing reagents for colony PCR. Prepare the colony PCR mixture according to the reaction table below. It is highly recommended to make a batch reaction mix (Master Mix) for efficient, error-free set-up of multiple colony PCR reactions. Multiply the volume of each reagent by the number of colonies to be screened plus one to account for pipetting error ($n + 1$).

A	B	C
Primer	**Sequence**	**Purpose**
M13 f	GTAAAACGACGGCCAG	Forward primer for PCR-Blunt II-TOPO (5′ EcoRI site), and GGDest1-Amp, GGDest2-Amp, and GGDest3-Amp (5′ BbsI site).
M13 r	CAGGAAACAGCTATGAC	Reverse primer for PCR-Blunt II-TOPO (3′ EcoRI site), and GGDest1-Amp, GGDest2-Amp, and GGDest3-Amp (3′ BbsI site).
GGDestX1-Amp F	GTGATAATGGTTGCAGCTAGC	Forward primer for GGDestX1-Amp (5′ BbsI site).
GGDestX1-Amp R	GGCTTTGCTCGAGTTAGC	Reverse primer for GGDestX1-Amp (3′ BbsI site).
GGDestX2-Amp F	TTTCAGGTTGGACCGGTG	Forward primer for GGDestX2-Amp (5′ BbsI site).
BGHR	TAGAAGGCACAGTCGAGG	Reverse primer for GGDestX2-Amp (3′ BbsI site).
U6	GACTATCATATGCTTACCGT	Forward primer for GGDestX2-Amp (U6 promoter, at 5′ of gRNA drop-in site).

TABLE 2 | Primer sequences for recombinant DNA analysis with colony PCR, gel electrophoresis, and Sanger sequencing.

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A	B	C	D
Reagent	**Volume per Colony (µL)**	**Master Mix**	**Final Concentration**
Forward primer 10 µM	1.0	1.0 x (n + 1)	0.4 µM
Reverse primer 10 µM	1.0	1.0 x (n + 1)	0.4 µM
DreamTaq Green PCR Master Mix (2X)	12.5	12.5 x (n + 1)	1X
MB H2O	10.5	10.5 x (n + 1)	
Total	25.0	25.0 x (n + 1)	

11 **Colony PCR**. 1h 12m

11.1 5m

Label and pre-warm an agar plate. Label the bottom surface of an LB agar plate (with the appropriate selection antibiotic) with a numbered grid (labeled 1, 2, 3 … *n*) to accommodate streaks for all colonies that will be screened. Incubate the plate at **37 °C** until you are ready to use it at Step 11.3.

11.2 5m

Pipette **25 µl** of PCR Master Mix into one PCR tube per colony (labeled 1, 2, 3 … *n*).

11.3 2m

Pick a colony from the ligation plate with a clean sterile micropipette tip or inoculation needle, streak the colony on the agar plate in numbered square within the grid, and gently swirl the same tip or inoculation needle in the corresponding PCR tube with Master Mix.

11.4 1h

Run the following thermocycler program.

A	B	C	D
Cycle Number	**Denature**	**Anneal**	**Extend**
1	95°C, 5 min		
2-36	95°C, 15 s	*#°C, 15 s	72°C, 30 s
37			72°C, 3 min

*Use the appropriate annealing temperature appropriate for the primer pair.

12 45m

Agarose Gel Electrophoresis. Cast a 1% (wt/vol) low EEO agarose gel in 1X TAE buffer with 1:10,000 SYBR Safe DNA Gel Stain. Load **3 µl** of GeneRuler 1 kb DNA ladder in the first well and **15 µl** of each colony PCR product in the remaining wells. Run the gel at 110 V for **00:45:00** .

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13 **Gel Imaging and confirmation of colony PCR amplicons**. 18h 10m

13.1 10m

Remove the gel from the electrophoresis chamber and use an imager to record the results. Compare the observed results to the expected length(s) of the PCR products. Positive clones should yield a single product the length of the donor fragment plus the distance of the primer to the cloning site.

13.2 18h

Place the streak plate from Step 11.3 in a **37 °C** incubator **Overnight** . The next day, seal the plate with Parafilm and store it at **4 °C** . Bacteria from streaks for confirmed clones will be used to inoculate new liquid cultures later.

Recombinant Plasmid Preparation and Verification with Restriction Digests 2d 20h 15m

14 18h

Liquid cultures of colony PCR-confirmed plasmid donor colonies. Obtain the colony streak plate and identify the streaks confirmed with colony PCR. Prepare 14 mL liquid culture tubes for the colonies to be grown by pipetting **3 mL** to **5 mL** of growth medium (supplemented with the appropriate antibiotic) into each tube. Pick bacteria from the streaks with sterile pipette tips and place the tips into their respective tubes. Place tubes on a shaking rack in a **37 °C** incubator for **07:00:00** (NEB DH5α-Turbo) to **18:00:00** (other strains, e.g. BL21).

15 1h

Plasmid DNA extraction. Isolate the plasmid DNA from the cultures by using the GenElute™ PCR Clean-Up Kit according to the manufacturer's instructions.

16 30m

Restriction digest of plasmid DNA minipreps. For immediate verification of successful ligations, digest a small amount of each miniprep according to **Table 3**. For efficient, error-free set-up of several reactions, prepare a restriction digest Master Mix. Multiply the volume of each reagent by the number of minipreps to be screened plus one ($n+1$). Pipette **13 µl** of the restriction digest Master Mix into 0.5 mL PCR tubes (labeled 1, 2, … n). Add **2 µl** of each DNA miniprep into the respective PCR tube. Tap the tubes gently to mix and incubate the reactions at **37 °C** for **00:05:00** to **00:30:00** .

A	B	C
Vector	**FD Enzyme(s)**	**Empty vector fragment(s) (bp)**
Donor plasmid (Zero Blunt™ TOPO™)	EcoRI	3501, 18
GGDest1-Amp	EcoRI, BcuI (SpeI)	3245, 57
GGDest2-Amp	BamHI, HindIII	3243, 36
GGDest3-Amp	NdeI, LguI (SapI)	2936, 314, 34
GGDestX1-Amp	NdeI, XhoI	2523, 707
GGDestX2-Amp	EcoRI, XbaI	3433, 856

TABLE 3 | FastDigest restriction enzymes for verifying recombinant plasmids.

Citation: James H Priode, Karmella A Haynes (01/19/2021). Rapid Single-Pot Assembly of Modular Chromatin Proteins for Epigenetic Engineering. https://dx.doi.org/10.17504/protocols.io.brgcm3sw

A	B	C	D
Reagent	**Volume per Miniprep (µL)**	**Master Mix**	**Final Concentration**
Miniprep DNA	2.0	0	(50 - 500 ng)
FastDigest Green Buffer (10X)	1.5	1.5 x (n + 1)	1X
*Enzyme(s)	2.0	1.0 x (n + 1)	
MB H2O	10.5	10.5 x (n + 1)	
Total	15.0	13.0 x (n + 1)	

*If using one enzyme instead of two, use 1.0 µL enzyme and 1.0 µL MB H_2O.

17 45m

Gel electrophoresis of digested DNA. After the restriction digest is complete, run a sample of the digest on a gel to verify the length of the sequence ligated into the vector. Cast a 1% (wt/vol) agarose gel in TAE buffer with SYBR DNA gel stain. Load **3 µl** of GeneRuler 1 kb DNA ladder into the first well and **15 µl** of digest product into the next wells. Run the gel at 100 V for **00:45:00** . Successful ligation of the correct insert sequence into the donor vector should yield a length of the full length insert plus the distance of the enzyme cut sites to the insert.

18 2d

Validation of plasmids via Sanger Sequencing. Verify the sequence of the isolated plasmids by sequencing using the primer pair appropriate for the vector (**Table 2**). The resulting sequences should be compared to a reference sequence for the expected plasmid product.

Golden Gate Assembly of Fusion Protein Plasmids 3d 10h 10m

19 25m **Preparation of donors and destination vector for single-pot assembly**. The Golden Gate cloning method for single-pot assembly requires a 1:1 molar ratio of each insert donor to the destination vector, with a recommended amount of 1 µl each for the reaction.

19.1 10m

Use a spectrophotometer to measure the concentrations (ng/µl) of donor and destination vector DNA stock.

19.2 5m

Determine the starting molarity of each stock DNA:

dsDNA fmol/µL = (dsDNA ng/µl / ((length of dsDNA bp x 617.96 ng/nmol/bp) + 36.04 ng/nmol)) / 10^6

Note: New England Biolabs provides a tool, the NEBioCalculator (https://nebiocalculator.neb.com/#!/dsdnaamt), for converting DNA length to mass.

19.3 10m

Make a working solution of **50 µl** at 40 fmol/µL for each donor DNA and destination vector as shown for the examples in the table below.

Citation: James H Priode, Karmella A Haynes (01/19/2021). Rapid Single-Pot Assembly of Modular Chromatin Proteins for Epigenetic Engineering. https://dx.doi.org/10.17504/protocols.io.brgcm3sw

A	B	C	D	E	F
Sample	**Stock DNA**			**Working solution: DNA**	**Working solution: H2O**
	Length (bp)	**ng/µL**	**Calculated fmol/µL**	**DNA µL**	**MB H20 µL**
Donor A	200	50	404.5	4.9	45.1
Donor B	200	200	1618	1.2	48.8
Donor C	1000	50	80.91	24.7	25.3
Donor D	1000	200	323.6	6.2	43.8
Destination Vector	3500	300	138.7	14.4	35.6

20  10m

Golden Gate reaction set-up. Prepare the reaction(s) according to the table below, with a final volume of **10 µl** per reaction.

A	B	C
Reagent	**Volume (µL)**	**Final Amount or Concentration**
Donor A	1.0	40 fmol
Donor B	1.0	40 fmol
Donor C	1.0	40 fmol
Donor D	1.0	40 fmol
Destination Vector	1.0	40 fmol
T4 DNA Ligase Buffer with 10 mM ATP (10X)	1.0	1X Buffer, 1 mM ATP
*Quick Ligase (T4)	0.5	
FastDigest BpiI (BbsI)	0.5	
MB H2O	3.0	
Total	10	

*Reagent from the NEB Quick Ligation™ Kit.

21  4h

Golden Gate assembly reaction. Run the single-pot assembly reaction with the following thermocycler program:

A	B	C	D
Cycle Number	**Digestion**	**Ligation**	**Heat Inactivation**
1-25	45°C, 2 min	16°C, 5 min	
26	60°C, 10 min		
27			80°C, 20 min

22 **Quick transformation**. Transform the Golden Gate reaction product into a competent *E. coli* strain, using guidelines appropriate for the strain. We recommend NEB DH5α-Turbo. Quick transformation (skipping outgrowth in SOC) is possible for plasmids that carry an ampicillin resistance marker, i.e. the GGDest series described in the guidelines. 7h 10m

22.1  10m

Citation: James H Priode, Karmella A Haynes (01/19/2021). Rapid Single-Pot Assembly of Modular Chromatin Proteins for Epigenetic Engineering. https://dx.doi.org/10.17504/protocols.io.brgcm3sw

Combine **5 µl** of the product with **50 µl** of chemically competent cells and let incubate on ice for **00:10:00** .

22.2 7h

Pipette the transformed cells onto a warmed agar plate (100 µg/mL ampicillin). Spread the cells with plating beads and place in a **37 °C** incubator for at least **07:00:00** for DH5α-Turbo cells. Other strains may require overnight incubation.

23 2h 10m

Plasmid screening with colony PCR. Follow the steps described under “Recombinant Plasmid Screening With Colony PCR” to identify 4 - 6 candidate clones with full-length inserts.

24 2d 20h 15m

Recombinant plasmid preparation and verification. Follow the steps described under “Recombinant Plasmid Preparation and Verification with Restriction Digests.” It is important to include Sanger Sequencing (one forward reaction and one reverse reaction per construct) to determine if modules A - D were assembled in the correct order.

Customization of Target-Specific gRNA in GGDestX2-Amp plasmids 3d 7h 15m

25 2h 10m **Design and produce gRNA dsOligos.** If you used GGDestX2-Amp to build a dCas9 fusion protein in the previous steps, use the following procedure to customize the guide RNA sequence.

25.1 1h

Follow the guidelines from Ran et al. to identify a 20 bp target site near the gene or within a genomic region of interest [52]. We recommend the Benchling CRISPR design tool (https://www.benchling.com/crispr/) for target detection and scoring.

25.2 10m

Design ssOligos according to **Table 4** below to produce a double-stranded oligo that is specific to the 20 bp genomic target site of interest and has single-stranded overhangs that are compatible with the BsaI-generated ends in GGDestX2-Amp.

A	B	C
ssOligo	**ssOligo Sequence (5’-3’)**	**Description**
gRNA top	CACCNNNNNNNNNNNNNNNNNNN	CACC = BsaI overhang, N20 = gRNA target sequence
gRNA bottom	AAACNNNNNNNNNNNNNNNNNNN	AAAC = BsaI overhang, N20 = reverse complement of target sequence

TABLE 4 | ssOligos used to build dsOligos for Type IIS drop-in.

25.3 10m

Set up reactions for phosphorylation and annealing of ssOligos as shown in the table below.

Citation: James H Priode, Karmella A Haynes (01/19/2021). Rapid Single-Pot Assembly of Modular Chromatin Proteins for Epigenetic Engineering. https://dx.doi.org/10.17504/protocols.io.brgcm3sw

A	B	C
Reagent	**Volume (µL)**	**Final Amount or Concentration**
100 µM gRNA top ssOligo	1.0	10 µM ssOligo
100 µM gRNA bottom ssOligo	1.0	10 µM ssOligo
T4 ligation buffer (10X)	1.0	1X T4 ligation buffer
T4 PNK	0.3	
MB H2O	6.7	
Total	10	250X dsOligo (10 µM)

25.4

50m

Run the ssOligo phosphorylation and annealing reaction(s) with the thermocycler program shown in the table below. Dilute **1 µl** of the resulting 250X dsOligo solution in **249 µl** MB H_2O to make a 1X working solution for Step 26.

A	B	C	D	E
Cycle Number	**Phosphorylation**	**PNK Deactivation & DNA melting**	**DNA Annealing**	**Hold**
1	37°C, 30 min			
2		95°C, 5 min		
3			Ramp down at 5°C per min to 25°C	
4				4°C, ∞

26

10m

DsOligo drop-in reaction set-up. After you have verified a GGDestX2-Amp clone that has a properly assembled dCas9 fusion fragment, prepare Type IIS digestion T7 ligation reaction(s) as shown in the table below, with a final volume of **10 µl** per reaction.

A	B	C
Reagent	**Volume (µL)**	**Final Amount or Concentration**
Recombinant GGDestX2-Amp plasmid	≤ 6.3 µL	50 ng
dsOligo (1X) (40 nM)	1.0	4 nM dsOligo
Fermentas Buffer G (10X)	1.0	1X Fermentas Buffer G
10 mM DTT	0.5	0.5 mM DTT
10 mM ATP	0.5	0.5 mM ATP
BsaI (Eco31I)	0.5	
T7 DNA ligase	0.2	
MB H2O		Bring final volume to 10 µL
Total	10	

27

1h

DsOligo drop-in reaction. Run the digestion-ligation reaction(s) with the following thermocycler program:

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A	B	C	D
Cycle Number	**Digestion**	**Ligation**	**Hold**
1-6	37°C, 5 min	23°C, 5 min	
7			4°C, ∞

28 30m

PlasmidSafe treatment. Treat ligations with PlasmidSafe exonuclease to prevent unwanted recombination products. Incubate at **37 °C** for **00:30:00** .

A	B	C
Reagent	**Volume (µL)**	**Final Amount or Concentration**
Digestion-ligation reaction	3.7	
PlasmidSafe Buffer (10X)	0.5	1X PlasmidSafe Buffer
10 mM ATP	0.5	1 mM ATP
PlasmidSafe exonuclease	0.3	
Total	5	

29 7h 10m

Transformation. Transform **50 µl** of chemically competent *E. coli* (e.g. DH5α-Turbo) with all **5 µl** of the PlasmidSafe reaction. Follow the quick transformation procedure described in Steps 22.1 - 22.2.

30 2d 20h 15m

Recombinant Plasmid Preparation and Verification. Follow the steps described under "Recombinant Plasmid Preparation and Verification with Restriction Digests" to extract and purify plasmid DNA from 2 - 4 colonies, but do not perform the restriction digest. Use Sanger Sequencing (U6 primer, **Table 2**) to determine if the gRNA region contains the appropriate custom target sequence. Although the background colony count (from a vector-only digestion-ligation control) is usually high, the gRNA drop-in is extremely efficient. In our hands a desired clone can often be found by picking just one or two colonies.

Synthetic Chromatin Protein Expression in vitro for GGDestX1-Amp Plasmids 19h 15m

31 10m

The following procedure is for fusion proteins that include a fluorescent protein tag and have been constructed in GGDestX1-Amp, which carries an Or2-Or1-Pr promoter that enables strong expression in cell-free reactions [53]. For non-fluorescent proteins, additional guidelines for a Bradford protein quantification assay are provided.

Prepare DNA templates. Prepare working solutions of 45 ng/µL for the recombinant plasmid (built from GGDestX1-Amp) and a GGDestX1-Amp (or P70a(2)-deGFP) control. The expected yield for preparations of P70a, GGDestX1-Amp and their derivatives is low because the backbone carries a ColE1 origin of replication (15 - 20 plasmid copies per cell).

32 10m

TXTL reaction set-up. Prepare reactions in a 96-well qPCR plate. Test each plasmid at 62.5 and 125.0 ng/well at minimum. Test additional dilutions when comparing rates of expression over several template concentrations [53]. Set up each unique reaction in triplicate wells by first adding the template DNA (≤ **3 µl**), the amount of MB H_2O needed

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to bring the final reaction to **12 µl** , then the myTXTL reagent. Include at least one mock (negative control) well. A single mock control well is sufficient if the amount of myTXTL reagent is in limited supply.

A	B	C	D
Reagent	**Volume (µL)**		
	125 ng DNA	**62.5 ng DNA**	**0 ng* (mock)**
myTXTL reaction buffer	9	9	9
Template DNA (45 ng/µL)	2.8	1.4	0
MB H2O	0.2	1.6	3
Total	12	12	12

33 17h

TXTL reactions in a real-time thermocycler. Run the TXTL reaction for 96 10-min cycles at **29 °C** . For each cycle, include a signal detection step (record) to measure expression of the recombinant plasmids. Use a dye-detection channel that is appropriate for the type of fluorescent protein that is encoded in the plasmid (for examples see the table below).

A	B	C
Cycle	**Hold**	**Record**
1-96	29°C, 9:59 min	29°C, 1 sec

A	B	C	D
Fluorescent protein	**Emission (nm)**	**Dye detection channel**	**Emission (nm)**
EGFP (P70a or GGDestX1-Amp)	509	*SYBR	520
EGFP (P70a or GGDestX1-Amp)	509	FAM	517
AmCyan	491	FAM	517
Venus	528	SYBR	520
mCherry	610	*Texas Red	604
mCherry	610	ROX	603

Channels are shown for the QuantStudio 6 thermocycler. *Settings used for the data shown in Figure 6.

34 1h

Data analysis. Download the raw data (time versus fluorescence signal) from the real time thermocycler. Use software (e.g. Microsoft Excel or GraphPad Prism) to plot time on the x-axis and mean fluorescent signal and standard deviation on the y-axis. For each series determine the (1) maximum output signal and the (2) maximum protein production rate (slope of the trend if linear, Hill slope if nonlinear, see **Fig. 6**).

35 **Bradford assay**. A Bradford assay, or similar protein quantitation assay can be used to support the results from fluorescent signal detection, or if the synthetic chromatin fusion protein has no detectable fluorescence. 55m

35.1 10m

Prepare the following samples in a final volume of **5 µl** in MB H_2O: background (**5 µl** MB H_2O), BSA standards (0.125, 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 mg/mL), experimental TXTL samples (10 - 100% v/v), and mock TXTL (10 - 100% v/v).

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35.2 10m

Combine **5 µl** diluted sample and **250 µl** Bradford reagent in each well of a microwell plate. Set up triplicate wells for each sample where possible. We recommend testing diluted TXTL samples first and then increasing the concentration as needed.

35.3 5m

Incubate at room temperature for **00:05:00** and measure the absorbance at 595 nm in a microwell plate reader.

35.4 30m

Create a standard curve and linear regression using background-subtracted BSA standard values. Subtract the mock TXTL signal from the experimental TXTL values and use the equation from the linear regression to determine the protein concentration for each experimental sample.

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