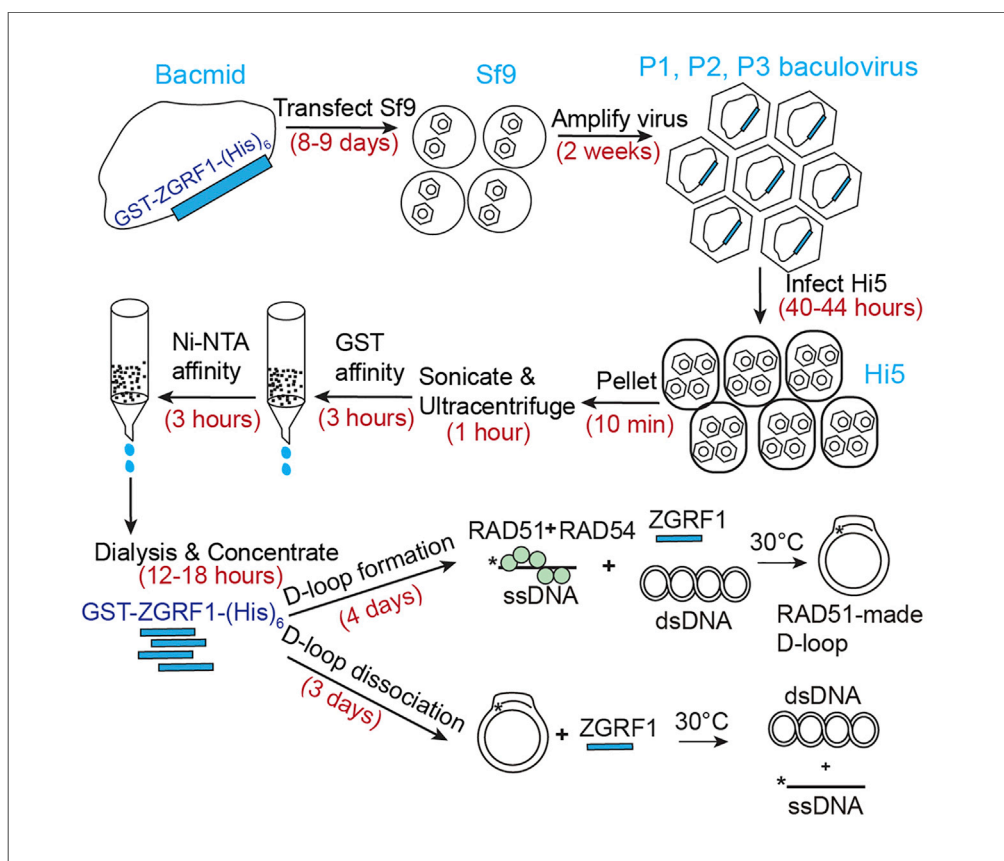


Protocol

Protocol for Purification of Human ZGRF1 and Its Regulatory Function on RAD51-Mediated D-Loop Formation



Human ZGRF1 is one of the many helicases that play important roles in the repair of damaged chromosomes by homologous recombination. However, the lack of biochemical characterization of the ZGRF1 protein has hindered the understanding of its function. We have developed a facile protocol to express human ZGRF1 in insect cells and for its purification. We have also tuned biochemical assays by lowering the protein and DNA concentrations to analyze its functions in RAD51-mediated D-loop formation and dissociation.

Michael Lisby,
Xiaoyu Xue

m lisby@bio.ku.dk (M.L.)
xiaoyu.xue@txstate.edu
(X.X.)

HIGHLIGHTS

Expression and purification of human ZGRF1 helicase in insect cells

Protocol to analyze ZGRF1's function in RAD51-mediated D-loop formation

Protocol to analyze ZGRF1's function in D-loop dissociation

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Protocol

Protocol for Purification of Human ZGRF1 and Its Regulatory Function on RAD51-Mediated D-Loop Formation

Michael Lisby^{1,2,*} and Xiaoyu Xue^{3,4,5,6,*}¹Department of Biology, University of Copenhagen, 2200 Copenhagen N, Denmark²Center for Chromosome Stability, Department of Cellular and Molecular Medicine, University of Copenhagen, 2200 Copenhagen N, Denmark³Department of Chemistry and Biochemistry, Texas State University, San Marcos, TX 78666, USA⁴Materials Science, Engineering, and Commercialization Program, Texas State University, San Marcos, TX 78666, USA⁵Technical Contact⁶Lead Contact*Correspondence: milisby@bio.ku.dk (M.L.), xiaoyu.xue@txstate.edu (X.X.)
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SUMMARY

Human ZGRF1 is one of the many helicases that play important roles in the repair of damaged chromosomes by homologous recombination. However, the lack of biochemical characterization of the ZGRF1 protein has hindered the understanding of its function. We have developed a facile protocol to express human ZGRF1 in insect cells and for its purification. We have also tuned biochemical assays by lowering the protein and DNA concentrations to analyze its functions in RAD51-mediated D-loop formation and dissociation.

For complete details on the use and execution of this protocol, please refer to Brannvoll et al. (2020).

BEFORE YOU BEGIN

Human ZGRF1 (zinc finger GRF-type containing 1, C4orf21) gene encodes a putative DNA/RNA helicase that promotes the removal of replication-blocking DNA lesions (Brannvoll et al., 2020). ZGRF1 protein is composed of 2,104 amino acid residues, and it contains a DUF2439 domain, a (GRF-type) Zn-finger domain, and a helicase domain. Purification of ZGRF1 to a degree suitable for detailed biochemical analysis has not yet been achieved. Given this, the role that ZGRF1 fulfills in HR and replication fork repair has until now not been investigated. Given that many tumors either experience heightened endogenous DNA damage and replication stress or are compromised for DNA repair, biochemical characterization of ZGRF1 may set the stage for developing chemical inhibitors as potential cancer therapeutics.

We have developed a unique protocol to express GST-(TEV)-ZGRF1-(His)₆ in Hi5 insect cells. The original human ZGRF1 cDNA (C4orf21-pCMV6) was purchased from Origene. We have also developed an efficient purification procedure in order to obtain a good yield of monodispersed protein. Furthermore, with purified RAD51 recombinase and its cofactor RAD54 in hand, we have optimized our RAD51-mediated D-loop to determine ZGRF1's effect on catalysis of the D-loop reaction. A D-loop dissociative activity of ZGRF1 has also been uncovered.

Primer Design

© Timing: 10 min



1. Primers to clone full-length ZGRF1 into pENTR™/TEV/D-TOPO™ vector, while introducing a C-terminal (His)₆ tag to ZGRF1. The QuikChange primers to introduce an ATPase-deficient version of the ZGRF1 (ZGRF1-K1660A) are also provided:
 - a. Forward: 5'-CACCATGGAAAGCCAAGAATTTATTG-3'
 - b. Reverse: 5'-TTAATGATGATGATGATGATGTGAATGAGATTTATCTTTAGATTT-3'
 - c. K1660A_Forward: 5'-CATGGTGTGTTTGGAGCAGGAGCGAGTTACTTGCTGGCAGTGGTG-3'
 - d. K1660A_Reverse: 5'-CACCACTGCCAGCAAGTAACTCGCTCCTGCTCCAAACACACCATG-3'

The ZGRF1-(His)₆ sequence was transferred from the entry clone into the pDEST20 expression vector to express GST-(TEV)-ZGRF1-(His)₆.

2. Sequencing Primers, to verify the DNA sequence of ZGRF1.
 - a. Sequencing Primer 1: 5'-ATGGAAAGCCAAGAATTTATTGTTC-3'
 - b. Sequencing Primer 2: 5'-AAAGAGATAGTTTGGCATCTCACTA-3'
 - c. Sequencing Primer 3: 5'-ACTGGAAAAGGAGTATGAACAATCA-3'
 - d. Sequencing Primer 4: 5'-AACAGTAATCTATTTTCAGAAGATG-3'
 - e. Sequencing Primer 5: 5'-AGCCTGTTGAGTTTCAAGGACATCA-3'
 - f. Sequencing Primer 6: 5'-AACTGATGGCTTCTTTGCTGAGGCT-3'
 - g. Sequencing Primer 7: 5'-GCTCGACCTGGCATGGTTTTAAGTG-3'
 - h. Sequencing Primer 8: 5'-AAAGCATTGAAGAAGTGAAGGAACT-3'
 - i. Sequencing Primer 9: 5'-TCACAAGCCAATTCTATTGAGAACT-3'
 - j. Sequencing Primer 10: 5'-TCATGTGATGCAGCTGATTCACCAC-3'

Maintenance of Cell Cultures

3. Maintain the Sf9 cells in Sf-900™ III SFM Medium in a suspension culture at 27°C and 160 rpm/min, on an orbital shaking incubator. We split the Sf9 cells every 2–3 days. It is not recommended to have more than 30 passages for the Sf9 cells (2–3 months).
4. Maintain the Hi5 cells in ESF 921 Insect Cell Culture Medium in a suspension culture at 27°C and 160 rpm/min, on an orbital shaking incubator. We split the Hi5 cells every 2 days. It is not recommended to have more than 30 passages for the Hi5 cells (2 months)

Note: Sf9 cells could also be propagated in ESF 921 Medium. Sf9 cells (Expression Systems, LLC) could be used as an alternative for Sf9 (Invitrogen). Tni cells (Expression Systems, LLC) could be used as an alternative for Hi5 cells. However, Tni cells give lower yields of ZGRF1 protein compared with Hi5 cells.

Preparation of Other Recombinant Proteins

⌚ Timing: 1 week

5. Detailed protocol to purify human RAD51 was described (Zhao et al., 2018). Human RAD54 was expressed and purified to near homogeneity using the following procedures.
 - a. Grow *E. coli* Rosetta cells harboring the pET32-hRAD54 plasmid that expresses human RAD54 with N-terminal Trx-(His)₆-S-tag and C-terminal Flag tag in 10 Liter LB at 37°C until the A₆₀₀ reaches 0.7–0.8.
 - b. Induce protein expression by adding 0.1 mM IPTG for 16 h at 16°C.
 - c. Resuspend the cell pellet (~28 g) at 4°C in 90 mL K+150 buffer with 20 µg/mL protease inhibitor cocktail (Roche) and 1 mM PMSF.
 - d. Sonicate the suspension at 30 amplitude with a 1/2-inch diameter probe (Qsonica) for 10 s eight separate times on ice.

- e. Clarify the crude lysate at 100,000 × g for 90 min.
- f. Equilibrate a Q Sepharose column (14 mL) and a SP Sepharose column (14 mL) with 100 mL K+150 buffer.
- g. Load the clarified lysate onto the Q Sepharose column and collect the flow-through. Apply the flow-through fraction onto the SP Sepharose column and fractionate with a 100 mL gradient of 150–650 mM KCl in K buffer.
- h. Incubate the fractions containing human RAD54 with 2.5 mL Ni-NTA agarose (pre-equilibrated with K+150 buffer) at 4°C for 1.5 h.
- i. Transfer the resin suspension to a chromatography column and drain by gravity.
- j. Wash the resin with 50 mL K+300 buffer containing 20 mM imidazole.
- k. Wash the resin with 50 mL K+1000 buffer containing 1 mM ATP, 5 mM MgCl₂ and 20 mM imidazole.
- l. Wash the resin with 50 mL K+150 buffer containing 20 mM imidazole.
- m. Elute the RAD54 protein with 4 mL K+150 buffer containing 200 mM imidazole.
- n. Repeat the elution step three additional times.
- o. Combine the eluates and mix with 8 mL K buffer.
- p. Load the mixture to a 1 mL Mono S column and fractionate with a 30 mL linear gradient of 150–500 mM KCl in K buffer.
- q. Pool the RAD54 containing fractions (eluted at ~330–360 mM KCl) and concentrate in an Ultracentrifugal Filter Unit (30K). The concentrated protein was divided into small aliquots and stored at –80°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
<i>E. coli</i> strain DH5α	Lifetechnologies	Cat#18258012
<i>E. coli</i> strain One Shot TOP10	Thermo Fisher Scientific	Cat#C404010
<i>E. coli</i> strain Rosetta	Novagen	Cat#70954
<i>E. coli</i> strain DH10Bac	Invitrogen	Cat#10361012
Chemicals, Peptides, and Recombinant Proteins		
Complete protease inhibitor cocktail	Roche	Cat#25735720
Glutathione Sepharose 4B resin	GE Healthcare	Cat#17075605
Ni-NTA Agarose	QIAGEN	Cat#30230
LB Broth Media	Teknova	Cat#L9135
SF-900™ III SFM Medium	Thermo Fisher Scientific	Cat#12658027
ESF 921 Medium	Expression Systems	Cat# 9600101
Cellfectin II Reagent	Gibco	Cat#10362100
PfuUltra High-Fidelity DNA Polymerase AD	Agilent Technologies	Cat#600385
dNTP	Fisher Scientific	Cat#BP25644
Glutathione, reduced	Acros Organics	Cat#120000050
Imidazole	Sigma-Aldrich	Cat#56749-250G
Phenyl-methylsulfonyl fluoride (PMSF)	Fisher Scientific	Cat#ICN800263
Igepal CA-630	Fisher Scientific	Cat#ICN19859690
Bluo-Gal	Invitrogen	Cat# 15519028
Tetracycline HCl	Fisher Scientific	Cat# BP912-100

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Gentamicin Reagent Solution	Gibco	Cat#15710-064
Isopropyl-b-D-thiogalactopyranoside (IPTG)	Fisher Scientific	Cat# BP1620-10
Kanamycin	Fisher Scientific	Cat#BP906-5
T4 polynucleotide kinase	New England Biolabs	Cat#M0201L
[γ - ³² P]-ATP	PerkinElmer	Cat#BLU002Z250UC
Phenol-chloroform-isoamyl alcohol (25:24:1)	Fisher Scientific	Cat#BP17521-100
Chloroform	Acros Organics	Cat#423555000
Potassium phosphate monobasic (KH ₂ PO ₄)	Fisher Scientific	Cat#BP362500
Glycerol	Fisher Scientific	Cat#BP2294
Ethylenediaminetetraacetic acid (EDTA), 0.5M	Fisher Scientific	Cat#AAJ15694AP
Dithiothreitol (DTT)	Fisher Scientific	Cat#BP17225
Potassium chloride (KCl)	Fisher Scientific	Cat#BP366500
Tris Base	Fisher Scientific	Cat#BP15210
Magnesium chloride (MgCl ₂)	Fisher Scientific	Cat#BP214500
Orange G	Fisher Scientific	Cat#O26725
Bovine Serum Albumin (BSA)	Fisher Scientific	Cat#BP9700100
ATP	Fisher Scientific	Cat#BP41325
Calcium chloride (CaCl ₂)	Fisher Scientific	Cat#C79500
Phosphocreatine	Sigma-Aldrich	Cat#10621714001
Creatine phosphokinase	Sigma-Aldrich	Cat#10127566001
Critical Commercial Assays		
QIAprep Spin Miniprep Kit	QIAGEN	Cat#27106
pENTR™/TEV/D-TOPO™ Cloning Kit	Invitrogen	Cat#K252520
LR Clonase™ II enzyme mix	Invitrogen	Cat#11827-011
QuikChange II Site-Directed Mutagenesis Kit	Agilent	Cat#200523
Experimental Models: Cell Lines		
Sf9	Invitrogen; Expression Systems, LLC	n/a; Cat#94-001F
Hi5	Invitrogen	n/a
Tni	Expression Systems, LLC	Cat#94-002F
Oligonucleotides		
ZGRF1_Forward Primer	Integrated DNA technologies	n/a
ZGRF1_Reverse Primer	Integrated DNA technologies	n/a
K1660A_Forward Primer	Integrated DNA technologies	n/a
K1660A_Reverse Primer	Integrated DNA technologies	n/a
Sequencing Primers	Integrated DNA technologies	n/a
90-mer oligonucleotide	Integrated DNA technologies	(Raynard and Sung, 2009)
Recombinant DNA		
C4orf21-pCMV6	Origene	Cat#PS100001
pDEST20 vector	Invitrogen	Cat#11807013
ZGRF1-(His) ₆ -pDEST20 expression vector	This study	This study
pBluescript SK+	Fermentas GmbH, Germany	(Short et al., 1988)

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and Algorithms		
Quantity One	Bio-Rad	n/a
Other		
Gravity flow column (Poly-Prep Chromatography Columns)	Bio-Rad	Cat#7311550
Slide-A-Lyzer™ MINI Dialysis Device	Thermo Scientific Scientific	Cat#88405
Spin-X Ultrafiltration Concentrator	Corning	Cat#431480
Micro Bio-Spin P-6 Gel Columns	Bio-Rad	Cat#7326222
Whatman 3MM chromatography paper	GE Healthcare	Cat#3030153
Storage Phosphor Screen	GE Healthcare	Cat#28956476
C1000 Touch Thermal Cycler	Bio-Rad	Cat#1851148
Nanodrop One	Thermo Fisher Scientific	Cat# 13400518PR2
Qsonica sonicator	Qsonica	Cat#Q700
Pharos FX Plus Molecular Imager	Bio-Rad	Cat#1709460
Optima Max Ultracentrifuge	Beckman	n/a
Sorvall Lynx 6000 Centrifuge	Thermo Fisher Scientific	Cat#75006590
Sorvall Legend Micro 17 Microcentrifuge	Thermo Fisher Scientific	Cat#75002431
AKTA pure 25 L1 FPLC	GE Healthcare	Cat#29018224-PMO18
Q Sepharose	GE Healthcare	Cat#17051001
SP Sepharose	GE Healthcare	Cat#17072901
Mono S column	GE Healthcare	Cat#17516801
Ultracentrifugal Filter Unit (30K)	MilliporeSigma	Cat#UFC903024
Drybath	Thermo Fisher Scientific	Cat#88870002

MATERIALS AND EQUIPMENT

Reagents

Solution	Contents	Final Volume	Storage
Protein Lysis Buffer	20 mM KH ₂ PO ₄ , pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT, 500 mM KCl, 20 μg/mL protease inhibitor cocktail, 1 mM PMSF	100 mL	4°C, make fresh
Binding Buffer	20 mM KH ₂ PO ₄ , pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT, 500 mM KCl	1 L	4°C, 1–2 weeks
Glutathione Elution Buffer	Binding Buffer + 15 mM reduced glutathione	12 mL	4°C, 2 weeks
Imidazole Wash Buffer	Binding Buffer + 15 mM imidazole	25 mL	4°C, 2 weeks
Imidazole Elution Buffer	Binding Buffer + 200 mM imidazole	5 mL	4°C, 2 weeks
Dialysis Buffer	Binding Buffer	500 mL	4°C, 1–2 weeks
Buffer H	35 mM Tris-HCl pH 7.5, 1 mM DTT, 9.3 mM MgCl ₂ , and 30 mM KCl	50 mL	4°C, 2 weeks
Agarose gel loading buffer	20 mM Tris-HCl pH 8.0, 50% glycerol, with Orange G dye	1 mL	–20°C, years
TBE buffer	45 mM Tris-borate, 1 mM EDTA, pH 8.0	1 L	22°C–25°C, 1 month
T+150 buffer	25 mM Tris-HCl, 10% glycerol, 0.5 mM EDTA, 150 mM KCl	1 L	4°C, 3–4 weeks

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Continued

Solution	Contents	Final Volume	Storage
K buffer	20 mM KH ₂ PO ₄ , pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT	1 L	4°C, 1–2 weeks
K+150 buffer	20 mM KH ₂ PO ₄ , pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT, 150 mM KCl	1 L	4°C, 1–2 weeks
K+300 buffer	20 mM KH ₂ PO ₄ , pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT, 300 mM KCl	1 L	4°C, 1–2 weeks
K+1000 buffer	20 mM KH ₂ PO ₄ , pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT, 1,000 mM KCl	1 L	4°C, 1–2 weeks

STEP-BY-STEP METHOD DETAILS

Molecular Cloning of ZGRF1 into pDEST20 Expression Vector

⌚ Timing: 6 days

To express human ZGRF1 in insect cells, we first introduced the ZGRF1 cDNA into the pENTR™/TEV/D-TOPO™ vector, while introducing a C-terminal (His)₆ tag to ZGRF1. The entry clone was subsequently introduced into the pDEST20 expression vector. As such, ZGRF1 harbors an N-terminal cleavable GST tag as well as a C-terminal (His)₆ tag. The two tags enable the use of affinity matrices to greatly facilitate the protein purification process.

Note: GST-(TEV)-ZGRF1-(His)₆ in pDEST20 vector can be obtained from the authors upon request.

Generating a Baculovirus and Expressing GST-(TEV)-ZGRF1-(His)₆ in Insect Cells

⌚ Timing: 3–4 weeks

Sf9 and Hi5 insect cells are commonly used for protein expression. The major advantages of insect cell expression (compared to bacterial expression) are increased solubility, improved protein folding, and posttranslational modifications such as phosphorylation. This protein expression platform is particularly useful to express large proteins such as ZGRF1 and protein complexes. Once the ZGRF1-(His)₆-pDEST20 expression vector is generated, the first step is to produce a recombinant baculovirus, which is then amplified in Sf9 cells to produce a high-titer viral stock for protein expression. We have found that Hi5 cells give higher yields of ZGRF1 protein than Sf9 or Tni cells (Expression Systems).

1. Generate the recombinant bacmid DNA.
 - a. Use 10–25 ng of the ZGRF1-(His)₆-pDEST20 expression vector to transform DH10Bac competent *E. coli* cells using the standard procedure.
 - b. After transformation, add 1 mL SOC medium to the *E. coli* cells, shake the cells at 37°C at 200 rpm for 4 h.
 - c. Make a 5-fold and 25-fold dilution of the cells with SOC medium, plate 100 µL of each dilution on an LB agar plate containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL Bluo-gal, and 40 µg/mL IPTG.
 - d. Incubate plates for 48 h at 37°C. Transfer a white bacterial colony into 3 mL LB culture containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, and 10 µg/mL tetracycline.

△ **CRITICAL:** The incubation time in step 1d is critical. The LB plates need to be incubated at least 48 h at 37°C. Shorter incubation time may result in difficulties to distinguish between white and blue colonies.

- e. Incubate the culture in a 37°C shaking incubator for 12–16 h.
- f. Extract the bacmid DNA using QIAprep Spin Miniprep Kit according to manufacturer's instructions with the following modifications: (i) the 3 mL *E. coli* culture is resuspended, lysed, and neutralized with 0.3 mL buffers P1, P2, and 0.3 mL of 3 M potassium acetate, pH 5.5, respectively. (ii) Precipitate the bacmid DNA from the supernatant from the last step with 0.8 mL of isopropanol, centrifuge for 15 min at 14,000 × *g* to pellet bacmid DNA. (iii) Wash the pellet with 0.5 mL of 70% ethanol, remove the ethanol, and air dry the pellet for 10 min at 22°C–25°C.
- g. Dissolve the bacmid DNA in 40 µL of 1× TE Buffer, pH 8.0, for 12–16 h at 4°C. Mix gently by tapping the tube. The average concentration of bacmid DNA is about 2–3 µg/µL.

Note: The recombinant bacmid DNA could be amplified by PCR for sequencing using Sequencing Primers.

2. Use the recombinant bacmid DNA to transfect Sf9 cells to produce a low-titer virus.
 - a. Transfect Sf9 cells in a 6-well plate with the bacmid DNA using Invitrogen Cellfectin II Reagent according to manufacturer's instructions. Importantly, set up a negative control well without bacmid DNA.
 - b. After transfection, incubate the cells in a 27°C humidified incubator for 8–9 days until signs of viral infection are visible. On day 4–5 after transfection, add 0.5–1 mL fresh medium to support the growth of the cells and to prevent the wells from drying out. On day 8–9, the control Sf9 cells should become saturated in the well, while the transfected Sf9 cells should be larger in size and with lower confluence (~50% confluence).
 - c. Collect the primary virus (P1 viral stock) by centrifugation at 500 × *g* for 5 min to remove cells and debris. The P1 viral stock can be stored at 4°C for 2–3 months.
3. Amplify the viral stocks to generate a high-titer virus.
 - a. Grow Sf9 cells to the density of 2×10^6 cells/mL. Transfer 30 mL of cells into a 175 cm² cell culture flask. Swirl the flask gently and place it in a 27°C humidified incubator for 30 min to allow attachment of cells.
 - b. Add 1 mL of P1 viral stock into the Sf9 cell culture flask and swirl the flask gently.
 - c. Incubate the cells for 6–7 days in a 27°C humidified incubator or until about 40%–50% cells appear lysed.
 - d. Collect the 30 mL of the Sf9 cell culture medium and transfer to sterile 50 mL snap-cap tubes. Centrifuge the tubes at 500 × *g* for 5 min to remove cells and debris. This is the P2 viral stock and could be stored at 4°C for 2–3 months.
 - e. Repeat the process to generate the P3 viral stock.

△ **CRITICAL:** The P3 viral stock should be made fresh and used for protein expression within 2–3 weeks.

4. Express the recombinant GST-(TEV)-ZGRF1-(His)₆ in Hi5 cells.
 - a. An expression test experiment could be performed before large-scale expression by infecting 5 × 50 mL Hi5 cells (1×10^6 cells/mL) with 0.5, 1, 1.5, 2, 2.5 mL fresh P3 viral stock. Incubate the cells at 27°C at 170 rpm for 40–44 h. Harvest cells and perform an anti-GST western blot to determine an appropriate dilution of P3 viral stock for best expression.
 - b. For large-scale expression, inoculate 150 mL Hi5 cells into two 1,000 mL Erlenmeyer flasks and incubate at 27°C at 170 rpm. Maintain the cells for 1 day and expand to 300 mL (0.6×10^6 cells/mL) for each flask.

- c. It is important to perform viral infection when Hi5 cells are in the mid-logarithmic phase of growth at a density of 1×10^6 cells/mL. Infect the cells with 10–15 mL (or determined by the expression test) fresh P3 viral stock.
- d. Incubate at 27°C at 170 rpm for 40–44 h.

△ CRITICAL: The infection time here in step 4a and 4d is critical. Longer infection times may lead to aggregation of ZGRF1 and thus reduce the final yield of purified protein.

- e. After 40–44 h post infection, retrieve 10 µL of cell culture and examine it microscopically. The infected Hi5 cells should show an increase in cell diameter.
- f. Harvest cells at $500 \times g$ for 10 min at 4°C.
- g. Discard the supernatant and store the pellet at –80°C. Usually, 600 mL of the Hi5 cells will yield 7–8 g of cell pellet.

Purification of GST-(TEV)-ZGRF1-(His)₆

⌚ **Timing:** 2 days

Here, we describe the large-scale purification of the recombinant human ZGRF1 in Hi5 insect cells. The procedure exploits the GST tag at the N terminus and the (His)₆ tag at the C terminus of ZGRF1. The typical yield of the recombinant GST-(TEV)-ZGRF1-(His)₆ from 600 mL Hi5 cell culture is 50–100 µg. The GST tag could be removed by TEV protease if desired. All the purification steps are conducted at 0°C–4°C.

5. Preparation of cell lysate.
 - a. Prepare 100 mL Protein Lysis Buffer and keep on ice.
 - b. Thaw the frozen cells (~8 g) and resuspend cells in the chilled Protein Lysis Buffer.
 - c. Transfer the suspension into a beaker placed on ice.
 - d. Stir the mixture at 4°C for 15 min using a magnetic stirrer gently.
 - e. Sonicate the mixture at 50 amplitude with a 1/2 inch diameter probe, for 30 s three separate times on ice.

△ CRITICAL: It is critical to maintain the cell mixture at low temperature near 4°C. Keep the mixture on ice for at least 1 min after each 30 s sonication.

 - f. Clarify the crude lysate at $100,000 \times g$ for 30 min. Keep a small aliquot of the clarified lysate for further analysis.
6. Affinity purification based on the GST tag.
 - a. Pack 2 mL of Glutathione Sepharose 4B resin into a gravity column.
 - b. Wash the resin with 20 mL of ddH₂O.
 - c. Wash the resin with 20 mL of Binding Buffer.
 - d. Split the equilibrated resin into two 50 mL snap-cap tubes.
 - e. Mix the clarified cell lysate (~100 mL) with the affinity resin at 4°C for 1.5 h on a rotary platform at 40 rpm.
 - f. Collect the resin in a chromatography column, drain by gravity for about 20 min.
 - g. Wash the resin with 50 mL Binding Buffer, three times. Combine the wash and keep a small aliquot of wash for further analysis.

Optional: Wash the resin with 50 mL Binding Buffer plus 1 mM ATP, followed by 50 mL Binding buffer plus 5 mM MgCl₂ will allow the removal of additional protein contaminants, such as ATP-dependent chaperones.

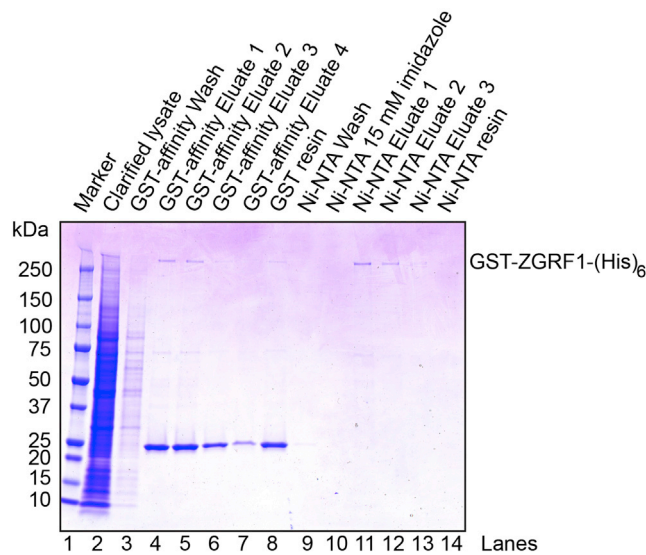


Figure 1. ZGRF1 Purification

Samples were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. Loading order from left: Marker, All Blue Prestained Protein Standards (Bio-Rad); GST-affinity Wash and Eluate, wash and eluate sample from GST resin; GST resin, GST resin after elution; Ni-NTA Wash and Eluate, wash and eluate sample from Ni-NTA resin; Ni-NTA 15 mM imidazole, 15 mM imidazole wash from Ni-NTA resin; Ni-NTA resin, Ni-NTA resin after elution.

- h. Elute GST-(TEV)-ZGRF1-(His)₆ with 3 mL Glutathione Elution Buffer at 4°C for 10 min by repeatedly passing the eluate through the packed resin.
 - i. Repeat the elution step three additional times with fresh Glutathione Elution Buffer. Keep a small aliquot of each eluate for further analysis.
 - j. After elution, take a small amount of the resin for further analysis.
7. Affinity purification based on the (His)₆-tag.
- a. Pack 0.5 mL of Ni-NTA Agarose resin into a narrow chromatography column.

△ CRITICAL: It is critical to use a small volume of Ni-NTA Agarose resin (~0.5 mL), as larger resin volume will result in lower concentration of ZGRF1 protein.

- b. Wash the resin with 5 mL of ddH₂O.
- c. Wash the resin with 5 mL of Binding Buffer.
- d. Transfer the resin to a new 15 mL snap-cap tube.
- e. Mix resin with the eluate (~12 mL) from the previous affinity purification step at 4°C for 2.5 h on a rotary platform.
- f. Transfer the resin suspension back to the chromatography column and drain by gravity.
- g. Wash the resin with 50 mL Binding Buffer, three times. Combine the wash and keep a small aliquot of wash for further analysis.
- h. Wash the resin with 25 mL Imidazole Wash Buffer. Keep a small aliquot for further analysis.
- i. Elute the GST-(TEV)-ZGRF1-(His)₆ protein with 0.6 mL Imidazole Elution Buffer at 4°C for 10 min by repeatedly passing the eluate through the packed resin.
- j. Repeat the elution with 0.6 mL fresh Imidazole Elution Buffer. Keep a small aliquot of each eluate for further analysis.
- k. After elution, take a small amount of the resin for further analysis.
- l. SDS-PAGE (4%–20%) analysis of the samples collected during the purification process (Figure 1).

Alternatives: an alternative procedure would be performing the affinity purification based on the (His)₆-tag followed by the affinity purification based on the GST tag.

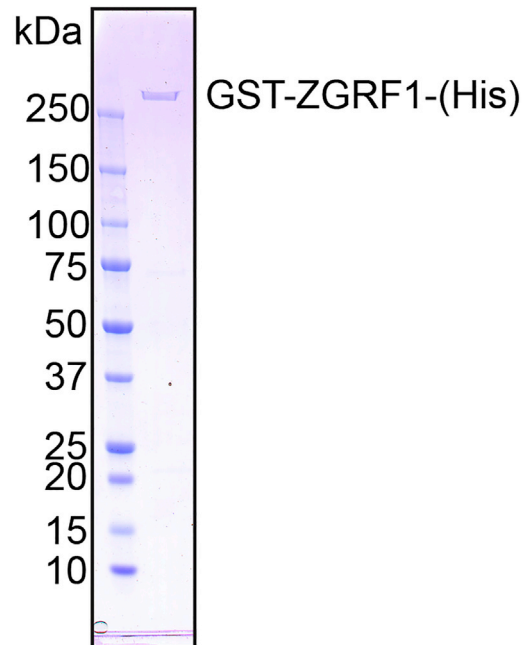


Figure 2. Final Purified and Concentrated ZGRF1

ZGRF1 final sample (4 μ L) was analyzed on SDS-PAGE and stained with Coomassie Brilliant Blue.

8. Dialysis and concentrate.

Note: Due to the small amount of purified ZGRF1 protein, we recommend dialysis devices to be used to reduce the loss of protein. [Troubleshooting 1](#).

- a. Add 44.5 mL Dialysis Buffer to a 50 mL conical tube and set aside.
- b. Add 4 mL of Dialysis Buffer into the Slide-A-Lyzer MINI Dialysis Device. Decant the buffer by shaking the device.
- c. Apply the combined protein sample (\sim 1.2 mL) into the dialysis device.
- d. Place the device slowly into the 50 mL conical tube containing the Dialysis Buffer. Make sure the membrane is in contact with buffer.
- e. Cap the conical tube securely and mix gently on an orbital shaker at 4°C.
- f. Continue dialysis for 2 h at 4°C.
- g. Change the Dialysis Buffer in the 50 mL conical tube.
- h. Continue dialysis for 12–14 h at 4°C.
- i. Collect the dialyzed protein sample. Apply the sample to a Corning Spin-X Ultrafiltration Concentrator (500 μ L, 50 kDa cutoff) to further concentrate to \sim 500 μ L.

△ CRITICAL: It is critical not to change buffer using Ultrafiltration Concentrator repeatedly, as this will cause aggregation of ZGRF1 protein on the membrane. Only concentrate it once to about 500 μ L.

- j. Split the purified ZGRF1 protein into 5 μ L aliquots, freeze in liquid nitrogen, and store at -80°C . ZGRF1 remains stable at -80°C for several years.
- k. Analyze the purified ZGRF1 by SDS-PAGE (4%–20%) ([Figure 2](#)).

Regulation of Human RAD51-Mediated D-Loop Formation Assay

© Timing: 4 days

ZGRF1 knockdown or knockout impairs HR efficiency, indicating its role therein (Adamson et al., 2012, Brannvoll et al., 2020). A D-loop formed between ssDNA and a donor duplex DNA is a key intermediate in the repair of DNA double-strand breaks or damaged DNA replication forks by HR. D-loop formation is catalyzed by RAD51 and multiple accessory proteins, such as BRCA1-BARD1 (Zhao et al., 2017) and RAD54 (Zhao et al., 2019). Here, we describe an optimized protocol to investigate the regulatory function of ZGRF1 in the RAD51-mediated D-loop reaction. To accommodate the low concentration of purified ZGRF1, we used a concentration of RAD51, RAD54 and DNA 3–4 fold lower than what would normally be used.

9. Preparation of pBluescript SK+ plasmid DNA.
 - a. Prepare the pBluescript SK+ plasmid DNA using QIAGEN Plasmid Mini or Midi kit.
 - b. Examine the topological state of the plasmid DNA by 0.9% agarose gel electrophoresis.

△ **CRITICAL:** The D-loop formation efficiency is sensitive to the topological state of the duplex DNA. The supercoiled form of plasmid DNA should be the major form, ideally ≥90% of total DNA.

10. 5'-end Radiolabeling of the 90-mer oligonucleotide.
 - a. Purify the 90-mer oligonucleotide (5'-AAATCAATCTAAAGTATATATGAGTAAACTTGGTC TGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTT-3', ordered from IDT, complementary to positions 1,932–2,022 of pBluescript SK+ plasmid DNA) from a 10% polyacrylamide gel.
 - b. Measure the DNA concentration spectrophotometrically. Store the purified oligonucleotide at –80°C until use.
 - c. Add the following reagents in a 1.5 mL microcentrifuge tube on ice and mix.

90-mer Oligonucleotide (65 μM)	5 μL
T4 polynucleotide kinase buffer (10×)	7 μL
[γ- ³² P]-ATP, 10 mCi/mL	5 μL
T4 polynucleotide kinase (10 U/μL)	4 μL
ddH ₂ O	49 μL

- d. Incubate the labeling reaction at 37°C for 1 h.
 - e. Inactivate T4 polynucleotide kinase by incubating at 65°C for 20 min.
 - f. Remove unincorporated [γ-³²P]-ATP using Micro Bio-Spin P-6 Gel Columns (Bio-Rad) according to the manufacturer's instructions.
 - g. Measure the DNA concentration spectrophotometrically. Store the labeled oligonucleotide at –20°C (shielded) until use.
11. D-loop formation assay.

Note: Before conducting D-loop formation assays, it is important to ensure that the purified ZGRF1 protein does not contain endo- or exonuclease contamination. Briefly, incubate 0–0.5 μg ZGRF1 with 10 nM ³²P-labeled 90-mer oligonucleotide or dsDNA (of any sequence) at 30°C for 1 h in a reaction buffer containing 20 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, 50 mM KCl and 1 mM DTT. The reactions are then deproteinized by treatment with SDS (0.1%) and proteinase K (0.5 mg/mL) for 10 min at 30°C and then resolved in a 15% polyacrylamide gel in TBE buffer. Any change in band intensity and migration pattern of the ssDNA or dsDNA, or appearance of short DNA smear (~1–5 nucleotides) compared to the negative control indicate potential nuclease contamination.

- a. Prepare a master mix of D-loop reaction buffer in a 1.5 mL microcentrifuge tube on ice and mix.

Reagent	Final Concentration	Amount
Tris-Cl, pH 7.5 (350 mM)	35 mM	12.5 μ L
DTT (10 mM)	1 mM	12.5 μ L
BSA (10 mg/mL)	0.1 mg/mL	1.25 μ L
ATP (100 mM)	2 mM	2.5 μ L
MgCl ₂ (25 mM)	1 mM ^a	5 μ L
CaCl ₂ (25 mM)	1 mM	5 μ L
Phosphocreatine (750 mM)	15 mM	2.5 μ L
Creatine phosphokinase (1,500 U/mL)	30 U/mL	2.5 μ L
³² P-labeled 90-mer oligonucleotide (37.5 μ M nucleotides)	750 nM nucleotides	2.5 μ L
ddH ₂ O	n/a	47.75 μ L
Total	n/a	94 μL

^aFinal MgCl₂ concentration could be increased to 3 mM to ensure free Mg²⁺ ions in the solution without impacting the stimulation of D-loop formation by ZGRF1.

Note: The master mix in the above table is for ten reactions. After adding RAD51, RAD54, ZGRF1 and pBluescript SK+ plasmid DNA, the final volume of each reaction is 12.5 μ L.

- b. Assemble reaction mixtures in 1.5-mL microcentrifuge tube on ice.
- c. For each reaction, add 9.4 μ L master mix to a 1.5-mL microcentrifuge tube.
- d. Add 0.6 μ L human RAD51 protein (4.5 μ M) to a final concentration of 216 nM.
- e. Incubate the reactions for 10 min at 37°C to allow RAD51 to engage the ssDNA.
- f. Add 0.5 μ L human RAD54 protein (2.25 μ M) to a final concentration of 90 nM.
- g. Incubate at 25°C for 2 min.
- h. Add 1.0 μ L human ZGRF1 protein (500 nM) to a final concentration of 40 nM. Use 1.0 μ L Dialysis Buffer as negative control.
- i. Immediately add 1.0 μ L pBluescript SK+ plasmid DNA (600 μ M base pairs) to a final concentration of 48 μ M base pairs and mix.
- j. Incubate at 30°C for 5, 10, and 20 min.
- k. Add 12.5 μ L of 1% SDS and 2 μ L of proteinase K (10 mg/mL) to stop the reaction.
- l. Incubate at 37°C for 5 min to deproteinize the reaction.

△ CRITICAL: It is critical that the human RAD51 and RAD54 are active; do not freeze and thaw the proteins repeatedly.

12. Analysis of the D-loop product.
 - a. For each reaction, withdraw 8 μ L of the above deproteinized reaction mixture, and add 2 μ L of agarose gel loading buffer.
 - b. Resolve the mixture in a 0.9% agarose gel in TBE buffer at 130 mA for 90 min at 25°C.
 - c. Dry the gel onto Whatman 3MM chromatography paper in a gel dryer for 1 h at 80°C.
 - d. Subject the dried gel to phosphorimaging analysis. One example of ZGRF1's effect on RAD51-mediated D-loop formation is shown in [Figure 3. Troubleshooting 2](#).

Note: In this protocol, human RPA was not included in our reaction. However, addition of 400 nM RPA after RAD51-ssDNA filament formation (step 11e) does not change the ability of ZGRF1 to stimulate D-loop formation by RAD51-RAD54.

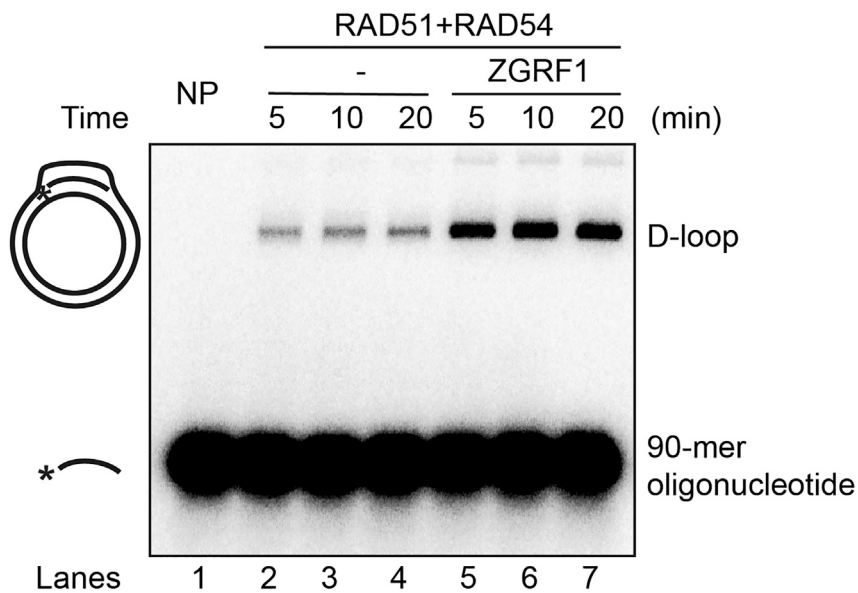


Figure 3. Promotion of RAD51-Mediated D-loop Formation by ZGRF1

Time course analysis of reactions catalyzed by RAD51 and RAD54, done in the absence or presence of ZGRF1. NP, no protein. *, 5'-³²P radio-label.

D-Loop Dissociation Assay

⌚ Timing: 3 days

To test whether ZGRF1 plays a role subsequent to D-loop formation, we have tested ZGRF1 on purified D-loops made by Rad51. We make D-loops and then remove all the proteins by phenol-chloroform extraction and further purify them in a desalting column.

13. D-loop generation.

- The yeast Rad51-mediated D-loop reaction (250 μ L) was performed with a ³²P-labeled 90-mer oligonucleotide as described (Xue et al., 2016). First, prepare a master mix of D-loop reaction buffer in a 1.5 mL microcentrifuge tube on ice.

Reagent (stock conc.)	Final Concentration	Amount
Tris-Cl, pH 7.5 (350 mM)	35 mM	25 μ L
DTT (10 mM)	1 mM	25 μ L
BSA (10 mg/mL)	0.1 mg/mL	2.5 μ L
ATP (25 mM)	2 mM	20 μ L
MgCl ₂ (125 mM)	5 mM	10 μ L
Phosphocreatine (750 mM)	15 mM	5 μ L
Creatine phosphokinase (1,500 U/mL)	30 U/mL	5 μ L
³² P-labeled 90-mer oligonucleotide (120 μ M nucleotides)	2.4 μ M nucleotides	5 μ L
ddH ₂ O	n/a	32.5 μ L
T+150 buffer	n/a	40 μ L
Total	n/a	170 μL

- b. Add 20 μ L yeast (*Saccharomyces cerevisiae*) Rad51 protein (10 μ M) to a final concentration of 0.8 μ M. Incubate the reaction for 10 min at 37°C.
- c. Add 20 μ L yeast RPA protein (2.5 μ M) to a final concentration of 200 nM. Incubate the reaction for 5 min at 30°C.
- d. Add 20 μ L yeast Rad54 protein (3 μ M) to a final concentration of 240 nM. Incubate the reaction for 2 min at 23°C.
- e. Add 20 μ L negatively supercoiled pBluescript SK+ plasmid DNA (600 μ M base pairs) to a final concentration of 48 μ M base pairs and mix. Incubate at 30°C for 4 min.
14. Phenol-chloroform extraction of yeast Rad51-made D-loops.
 - a. Add 4.5 μ L of 10% SDS and 10 μ L of proteinase K (10 mg/mL) to the above 250 μ L reaction mixture in a 1.5 mL microcentrifuge tube.
 - b. Incubate at 37°C for 15 min to deproteinize the reaction.
 - c. Add 260 μ L phenol-chloroform-isoamyl alcohol (25:24:1), mix thoroughly by vortexing.
 - d. Centrifuge at 22°C–25°C at 17,000 \times g for 5 min.
 - e. Transfer the upper aqueous phase into a fresh 1.5 mL tube.
 - f. Add 260 μ L chloroform, mix thoroughly by vortexing.
 - g. Centrifuge at 22°C–25°C at 17,000 \times g for 5 min.
 - h. Transfer the upper aqueous phase into a fresh 1.5 mL tube without any of the chloroform. Store this at 4°C for the next step.

△ CRITICAL: It is critical not to transfer any chloroform into the aqueous phase, as the organic solution will interfere with the D-loop dissociation reaction.

15. Further purification of D-loops.
 - a. Equilibrate the Zeba Spin-desalting Column (Thermo Scientific) with 15 mL Buffer H (35 mM Tris-HCl pH 7.5, 1 mM DTT, 9.3 mM MgCl₂, and 30 mM KCl).
 - b. Apply the above aqueous phase solution to the column without disturbing the surface of the column.
 - c. Collect the flow-through in 200 μ L portions into fresh 1.5 mL tubes.
 - d. Elute the column with Buffer H.
 - e. Collect the eluate in 200 μ L portions into fresh 1.5 mL tubes. Collect 15 such portions in total.
 - f. Determine the D-loop content in each fraction by gel electrophoresis in a 0.9% agarose gel in TBE buffer, followed by phosphorimaging analysis (Figure 4).

Note: The 90-mer oligonucleotides are copurified with the D-loops. Therefore, it is expected to have some 90-mer ssDNA in the fractions.

- g. Combine the eluates containing the bulk of D-loops and determine the concentration of the purified D-loops by side-by-side comparison with a known amount of the ³²P-labeled 90-mer oligonucleotide used for the D-loop reaction (Figure 4). [Troubleshooting 3](#).
16. D-loop dissociation by ZGRF1.
 - a. Prepare 8 μ L of D-loop dissociation buffer containing 25 mM Tris-Cl, pH 7.5, 1 mM DTT, 2 mM ATP, 2 mM MgCl₂ for each reaction, in a 1.5 mL microcentrifuge tube.
 - b. Add 1 μ L of ZGRF1 protein (2.5–20 nM) to each reaction and incubate on ice for 10 min.
 - c. Add 1 μ L of the purified D-loops (22 nM) to each reaction and mix. Note that the purified D-loop substrate contains about 55% free 90-mer ssDNA (Figure 5, lane 1).
 - d. Incubate at 37°C for 10 min.
 - e. To terminate the reaction, add 0.5 μ L of 10% SDS and 1 μ L of proteinase K (10 mg/mL) to the reaction mixture.
 - f. Incubate at 37°C for 5 min to deproteinize the reaction.
 - g. Add 3 μ L of agarose gel loading buffer.
 - h. Resolve reaction mixtures in a 0.9% agarose gel in TBE buffer at 130 mA for 90 min at 25°C.

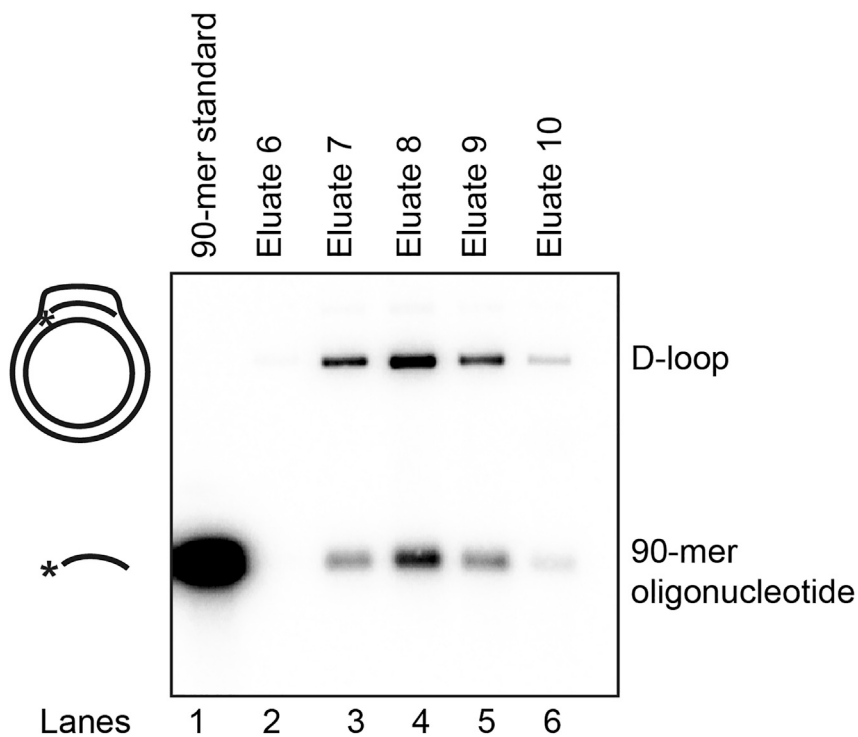


Figure 4. An Example of D-loop Purification

³²P-labeled 90-mer oligonucleotide standard, 10 ng of the same batch of radio-labeled 90-mer oligonucleotide; Eluate fractions 6–10, eluate from the desalting column with Buffer H, each eluate was ~200 μ L. Note that eluate 8 had the highest D-loop content (with some free 90-mer ssDNA copurified as expected) and was selected for examining D-loop dissociation by ZGRF1.

- i. Dry the gel onto Whatman 3MM chromatography paper in a gel dryer for 1 h at 80°C.
- j. Subject the dried gel to phosphorimaging analysis (Figure 5).

EXPECTED OUTCOMES

The N-terminal GST tag and the C-terminal (His)₆ tag greatly facilitate ZGRF1 purification. As shown in Figure 1, affinity purification making use of the GST tag yields not only ZGRF1, but also a significant amount of a 25 kDa protein. The second affinity purification step allows for ZGRF1 to be purified away from this contaminant or proteolytic product to near homogeneity (Figures 1 and 2). Furthermore, the dialysis step is designed to minimize the loss of ZGRF1 protein. Given that ZGRF1 may aggregate, it should not be concentrated beyond 500 nM. The final yield of highly purified ZGRF1 using this protocol should be 50–100 μ g per 600 mL cell culture. The procedures developed are also suitable for the expression and purification of ZGRF1 mutant forms.

The D-loop formation assay provides a powerful tool for investigating the presynaptic HR role of human ZGRF1. With the optimized protocol, we are able to clearly demonstrate that ZGRF1 enhances the RAD51-mediated D-loop formation (Figure 3 and Brannvoll et al., 2020). This biochemical result is consistent with our finding that knockout of ZGRF1 by CRISPR-Cas9 leads to a lower HR efficiency in cells as measured using the DR-GFP reporter (Brannvoll et al., 2020). The modified D-loop formation protocol should be applicable to studies involving other HR regulators.

We have found that ZGRF1 also possesses the ability to dissociate D-loops, similar with another DNA helicase/translocase FANCM (Figure 5 and Brannvoll et al., 2020). This suggests that ZGRF1 may help release the invading DNA strand from the D-loop intermediate in a major pathway of HR called synthesis-dependent strand annealing.

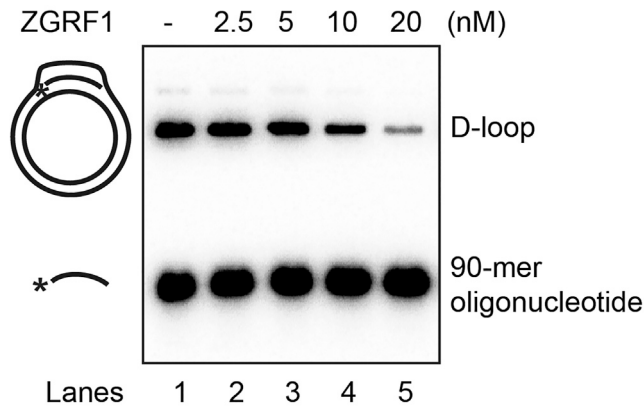


Figure 5. D-loop Dissociation by ZGRF1

Purified D-loops (~2.2 nM) were incubated with ZGRF1 (2.5–20 nM) at 37°C for 10 min. The reaction products were resolved in 0.9% agarose gels. Note the decrease of the D-loops and the slight increase in the free 90-mer ssDNA in lanes 2–5 compared with that in lane 1. The approximate percent increase in the 90-mer ssDNA should correspond to the percentage reduction in the D-loops.

LIMITATIONS

This protocol describes a method to purify the human ZGRF1 protein from insect cells. However, due to its tendency to aggregate at concentrations higher than 500 nM, we have not been able to generate highly concentrated ZGRF1 protein preparations. Since ZGRF1 interacts with human RAD51, FANCM and other proteins (Brannvoll et al., 2020), co-expression of the interacting proteins in insect cells may enhance the solubility of ZGRF1. The ZGRF1 protein purified using this protocol has a cleavable GST tag at its N terminus, which may interfere with some biochemical analyses. The solution is to cleave the GST tag off ZGRF1 during its biochemical analysis with the use of the TEV protease.

The modified D-loop protocol has enabled us to reveal a stimulatory effect of ZGRF1 in D-loop formation (Brannvoll et al., 2020). This sets the stage of testing possible synergy of ZGRF1 with other HR cofactors, such as BRCA1-BARD1, in the enhancement of D-loop formation.

TROUBLESHOOTING

Problem 1

Low yield of purified ZGRF1 (steps 5–8).

Potential Solution

1. Always use fresh P3 virus stock. When using the virus to infect Hi5 cells for expression, refrain from incubating the cell culture for longer than 44 h as ZGRF1 tends to aggregate and becomes proteolyzed with a longer incubation time.
2. The purification process should be completed within 24 h. Longer purification time may increase the possibility of protein aggregation and degradation.
3. Avoid concentrating the purified ZGRF1 protein beyond 500 nM as it tends to aggregate at higher concentrations.

Problem 2

Level of D-loop product is low (steps 11–15)

Potential Solution

1. Proteins lose their activity upon repeated freezing and thawing. Avoid this, and, once thawed, keep proteins on ice and test them within 48 h.

2. Test different concentrations of freshly prepared RAD51 and RAD54 proteins and vary the pH and salt concentration of the reaction mixture to improve reaction efficiency.
3. Ensure that the pBluescript SK+ plasmid DNA used is predominantly in the supercoiled form.
4. Check protein preparations for DNA nicking activity, which would reduce the content of the supercoiled form in the pBluescript SK+ plasmid DNA.

Problem 3

Low yield of purified D-loops (steps 13–15).

Potential Solution

1. Optimize the initial D-loop reaction to enhance the yield of D-loops.
2. Use only the purified fractions with the highest amounts of D-loops (Lane 4 in [Figure 4](#))

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Xiaoyu Xue (xiaoyu.xue@txstate.edu).

Materials Availability

All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and Code Availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

M.L. and X.X. conceived the project. X.X. performed experiments. X.X. and M.L. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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