Chapter 14

Purification and Characterization of RecQ Helicases of Plants

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Abstract

Helicases are essential for DNA metabolism. Different helicases have different properties tailored to fulfill their specific tasks. RecQ-helicases are known to be important in DNA repair and DNA recombination. In higher organisms several RecQ homologues can be identified. For instance, seven RecQ homologues were identified in the model plant *Arabidopsis thaliana*. Specialization of those proteins can possibly be reflected by differences in their biochemical substrate spectrum. Moreover, a helicase of interest might be defined by its biochemical properties as a functional ortholog of a RecQ helicase in other organisms. In this chapter the initial steps that will provide the basis for a proper biochemical characterization are given. After the description of the expression of the helicase of interest in the heterologous host *Escherichia coli*, its purification with the help of two affinity tags and the preparation of a model DNA substrate for the strand displacement assay are described. Finally, it is shown how this model substrate can be used to ensure the purity of the enzymatic preparation of interest.

Key words: RecQ, helicase, Walker A motif, *Arabidopsis thaliana*, Ni-IMAC, His-tag, overexpression, calmodulin binding peptide, calmodulin affinity chromatography, strand displacement assay.

1. Introduction

RecQ helicases play an important role in the maintenance of genomic stability and are conserved in all kingdoms of life (1–5). Whereas *Escherichia coli* possesses just one RecQ homologue, higher eukaryotes usually possess several RecQ homologues. In the model plant *Arabidopsis thaliana*, seven RecQ homologues were identified (6).

Several lines of evidence indicate a functional specialization of RecQ helicases (e.g., (7)). To some extent this specialization will also be reflected in different biochemical properties (e.g., (8)).

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Analyzing substrate preferences can help to assign a defined helicase to a specific DNA repair or DNA recombination pathway. In addition, comparing the biochemical properties of RecQ helicases helps to distinguish them and to define interspecies functional homologs.

In order to analyze the biochemical properties, first a protein preparation has to be obtained that is enzymatically active but free from contaminant activities. This chapter focuses on how this can be achieved, exemplified with the helicase activity of AtRECQ2 from *A. thaliana* (9). This protocol has already been successfully applied to other plant helicases.

Contaminating activities can be co-purified due to interactions of unwanted proteins with the column matrix, the affinity material, the target protein or by indirect interactions mediated, for instance, by DNA. Therefore the best control is to purify a protein that is as similar as possible to the protein of interest but does not show intrinsic activity, as it is the case for RecQ helicases with a specific amino acid substitution in the helicase motif I, which knocks out ATPase and thereby helicase activity (e.g., (10, 11)). Both protein preparations should be analyzed with all substrates as it is possible that another quite specific helicase or nuclease has been co-purified. A flow diagram is shown in **Fig. 14.1**.

The method described here exploits the T7 RNA polymerase expression system (12) and BL21-CodonPlus®(DE3)-RIPL. The expression vector used here allows the attachment of an

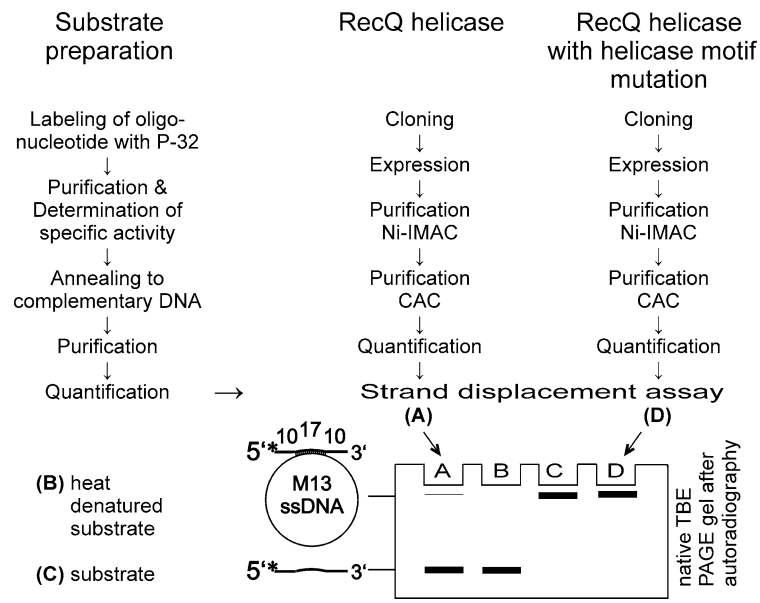

Fig. 14.1. Flow diagram of the basic steps toward the characterization of RecQ helicases of plants.

N-terminal calmodulin-binding peptide (for calmodulin affinity chromatography (13)), thrombin and enterokinase recognition sites, and a FLAG epitope (for detection in the Western blot) and at the C-terminus, a thrombin recognition site followed by an hexahistidine tag (for Ni-IMAC (14)). During expression, the correct folding of the target protein can be supported by reducing the temperature of expression (also *see* **Note 3**). The combination of the two affinity chromatography steps for purification selects full-length proteins and reduces the concentrations of contaminant proteins. A washing step with the detergent Triton-X-100 as well as a washing step of the Ni-IMAC with an optimized imidazole concentration remove contaminant proteins.

To determine if the helicase is functional and the preparation is free from contaminants, a DNA substrate providing a 3′ and a 5′ flap is used and a strand displacement assay is conducted (also *see* (15, 16)). If the helicase is active the duplex DNA region will be unwound, setting free the composing single-stranded DNA. The different DNA species can be separated via native gel electrophoresis. To provide a high sensitivity the substrate is labeled with P-32. For the strand displacement assay equal amounts of the helicase preparation (as well as dilutions of the presumable active enzyme) and the helicase with helicase motif I mutation are incubated with the substrate in a suitable buffer both in the presence and in the absence of ATP. The controls without ATP are well suited to judge contamination by nucleases. In case the RecQ preparation is enzymatically pure the characterization can be extended to more specialized DNA substrates, such as Holliday junctions, D-loops, or replication forks.

2. Materials

2.1. Expression of the Recombinant Plant Helicase in E. Coli

1. pCAL-n-FLAG (Stratagene) or another suitable vector
2. BL21-CodonPlus®(DE3)-RIPL (Stratagene) or another suitable expression strain
3. LB-medium: 10 g Trypton, 5 g yeast extract, 5 g NaCl, dissolve in ddH_2O, adjusted to 1 l, and autoclave
4. LB plates with ampicillin: LB medium with 1.5% (w/v) micro agar, autoclave, then cool down to approximately 50°C before adding ampicillin stock solution to a final concentration of 100 μg/ml, pour into petri dishes and store at 4°C
5. Antibiotic stocks: ampicillin (50 mg/ml in ddH_2O), carbenicillin (50 mg/ml in ddH_2O), chloramphenicol (50 mg/ml in ethanol)

6. 0.4 M isopropyl-ß-D-thiogalactopyranoside (Duchefa) in ddH_2O, filter to sterilize (0.22-µm PVDF filter)

2.2. Purification of the Recombinant Helicase by Double Affinity Purification

2.2.1. Preparation of the Soluble Protein Fraction for Affinity Chromatography

1. Buffer A: 20 mM Tris–HCl (pH 7.5), 200 mM NaCl, 20 mM imidazole, 5% glycerol, 10 mM 3-mercapto-1,2-propanediol (thioglycerol). Prepare all buffers for the purification without thioglycerol and filter through a 0.45-µm cellulose acetate filter (0.45-µm Sartorius AG) at 300 mbar with a vacuum pump. Additionally apply 300 mbar for at least 10 min to degas the solution. Store at 4°C.
2. Lysozyme (Roche Diagnostics).
3. Sonopuls Ultraschall-Homogenisator HD 2070 (Bandelin).
4. GF/PET filter (Roth).

2.2.2. Ni-IMAC

1. Buffer A as described in **Section 2.2.1**.
2. Buffer B: components as buffer A, plus 400 mM imidazole.
3. HiTrap chelating HP column (1 ml) (GE Healthcare).
4. Low pressure liquid chromatography system BioLogic LP (BioRad Laboratories).
5. 0.1 M $NiSO_4$ solution (in ddH_2O). Caution: toxicity.
6. PD-10 columns, filled with Sephadex G25-M (GE Healthcare).
7. Buffer C: 50 mM Tris–HCl (pH 7.5), 500 mM NaCl, 2 mM $CaCl_2$, 1 mM $Mg(CH_3COO)_2$, 1 mM imidazole, 10 mM thioglycerol.

2.2.3. Calmodulin Affinity Chromatography

1. Calmodulin affinity resin for purification of CBP-tagged proteins (Stratagene).
2. PolyPrep® chromatography column (0.8 × 4 cm) (BioRad Laboratories).
3. Buffer D: 50 mM Tris–HCl (pH 7.5), 500 mM NaCl, 2 mM EGTA, 10 mM thioglycerol.
4. Buffer E: 50 mM Tris–HCl (pH 7.5), 300 mM NaCl, 10% glycerol, 10 mM thioglycerol.
5. Buffer F: 50 mM Tris–HCl (pH 7.5), 1000 mM NaCl, 2 mM EGTA.
6. Buffer G: 0.1 M $NaHCO_3$, 2 mM EGTA (pH 8.6).
7. Buffer H: 1 M NaCl, 2 mM $CaCl_2$.
8. Buffer I: 0.1 M sodium acetate buffer, 2 mM $CaCl_2$ (adjust pH to 4.4. with acetic acid).
9. Servapor® dialysis tubing with 16 mm diameter, MWCO 12–14 kDa (Serva). Cut approximately 15 cm and put in ddH_2O with EDTA. Heat for at least 20 min at 70°C. Store at 4°C.

10. PD-10 columns, (filled with Sephadex G25-M) (GE Healthcare).

2.3. Preparation of the Radioactively Labeled DNA Substrate

1. Oligonucleotide 5′-AAAAAAAAAA GTC GAC TCT AGA GGA TC AAAAAAAAAA-3′; best results are obtained with PAGE purified oligonucleotides. The middle part of this oligonucleotide is complementary to nucleotides (nt) 6252–6268 of M13mp18.
2. [γ-^{32}P]ATP (3000 Ci/mmol) (GE Healthcare), GE Healthcare no longer manufactures this radioactive material. We now use Hartmann Analytic instead. A license is required to order this radioactive material as well as shielding.
3. T4 PNK and the T4 PNK buffer (New England Biolabs).
4. MicroSpin^{TM} G-25 Columns (GE Healthcare).
5. M13mp18 single-stranded DNA (New England Biolabs).
6. LumaSafe Plus (Lumac LSC) and scintillation vials.
7. Liquid scintillation analyzer Tri-Carb 2100 TR (Packard Instrument Company).
8. MicroSpin^{TM} S-400 HR columns (GE Healthcare) or alternatively Sephacryl^{TM} S-400 high resolution resin (GE Healthcare).

2.4. Detection of the Helicase Activity via the Strand Displacement Assay

1. TBE buffer (10 ×): 890 mM Tris-base, 890 mM boric acid, 20 mM EDTA, pH 8.0
2. Acrylamide (30% T, 2.67% C) (Roth)
3. N,N,N’,N’-tetramethylethylenediamine (TEMED)
4. 10% ammonium persulfate (APS) (w/v); dissolve in water, aliquot, and store at –20°C
5. Whatman Multigel-Long (Biometra) and connected cooling system
6. Reaction buffer (5 ×): 200 mM Tris–acetate, 250 mM potassium acetate (pH 8.0)
7. 120 mM DTT, dissolve in ddH_2O, aliquot and store at –20°C
8. 36 mM ATP (Fluka), calculate the necessary weight according the certificate of analysis (due to water in the chemical), dissolve in ddH_2O, aliquot, and store at –20°C
9. 1 M stock solution of $MgCl_2$ (Applichem) due to the hygroscopic behavior of $MgCl_2$. Dilute to 36 mM with ddH_2O
10. BSA (New England Biolabs) (10 mg/ml), dilute 1:10 with ddH_2O, aliquot, and store at –20°C
11. PCR chiller
12. Multichannel pipette
13. Heating block for PCR tubes

14. Stop solution (3 ×): 50 mM EDTA, 0.6% SDS, 20% glycerol, 0.1% xylene cyanol, 0.1% bromophenol blue
15. Saran Wrap (Roth)
16. Instant Imager (Canberra Packard Company)

3. Methods

3.1. Expression of the Recombinant Plant Helicase in E. Coli

1. Starting from the coding sequence information of the plant helicase of interest, identify (on the protein level) the helicase domain with its helicase motifs, e.g., by means of multiple sequence alignment with homologous helicases. Introduce a point mutation leading to an amino acid substitution for the conserved lysine of helicase motif I (also called Walker A) to methionine. We usually do this via overlap extension mutagenesis with the appropriately designed primers (17). Clone the ORF of interest and the ORF with the introduced point mutation in an expression vector for heterologous expression in *E. coli* and verify the construct by sequencing. Choose a vector that exploits the expression by T7 RNA polymerase system and allows the attachment of an N-terminal calmodulin binding peptide tag and a C-terminal His-tag to the protein of interest. For this purpose we modified the 3′ end of the MCS of the pCAL-n-FLAG vector (9).
2. Transform the constructs in competent BL21-CodonPlus®(DE3)-RIPL cells by heat shock transformation and plate on LB-plates containing ampicillin. Incubate overnight at 37°C. The following day, remove from the 37°C incubation and store at room temperature until the evening.
3. Inoculate 500 ml of LB with 75 µg carbenicillin/ml and 34 µg chloramphenicol/ml in a 1 l Erlenmayer flask with 50 colonies and incubate overnight at 37°C by means of shaking (200 rpm) (also *see* **Note 1**).
4. The next morning inoculate the appropriate number of 1 l Erlenmeyer flasks containing 500 ml of LB without antibiotics (*see* **Note 2**) with 50 ml of the cultures (helicase construct and helicase construct bearing point mutation, respectively) of step 3 and incubate at 28°C at 200 rpm. Monitor the optical density at 600 nm of the culture. If the optical density is between 0.6 and 0.9, add IPTG to a final concentration of 0.2 mM (this corresponds to 275 µl of a 0.4 M IPTG stock solution). Reduce the temperature to 16°C and continue to incubate by means of shaking at 200 rpm for approximately 20 h (*see* **Note 3**).

5. Harvest the *E. coli* cells by centrifuging at 2700 × g for 10 min and discard supernatant. It may be necessary to repeat the spinning process. At the end, transfer the pellet corresponding to 550 ml culture into one 50 ml polypropylene tube (by resuspending and centrifuging) and store it at –20°C.

3.2. Purification of the Recombinant Helicase by Double Affinity Purification

All purification steps are performed at 4°C or on ice.

3.2.1. Preparation of the Soluble Protein Fraction for Affinity Chromatography

1. Slowly thaw two identical cell pellets (from step 5 **Section 3.1**) on ice. Add 10 mM of the reducing agent 3-mercapto-1,2-propanediol (thioglycerol) (corresponds to 8.68 µl of thioglycerol per 10 ml of buffer) to the buffers and mix carefully. Carefully resuspend the cell pellets in 5 ml of buffer A, by pipetting over the pellet again and again. When this step has been completed, adjust the volume in each tube to 25 ml with buffer A making use of the scale on the tube.
2. Freshly prepare a lysozyme stock solution of 10 mg/ml in buffer A and add lysozyme to a final concentration of 0.1 mg/ml (corresponds to 250 µl of the stock solution). Incubate the cell suspension for 30 min on ice by means of shaking.
3. Break up the cells and disrupt the DNA in order to reduce viscosity by sonication. Use six 10-second cycles with 53% power und 50% duty cycle and between cycles cool down the extract on ice for 1 min.
4. Adjust the weight in ultracentrifugation tubes and ultra-centrifuge at 40,000 × *g* for 30 min at 4°C.
5. Combine the supernatants from the two pellets and filter through a GF/PET filter (*see* **Note 4**).

3.2.2. Ni-IMAC

Each chromatography column should be used for one construct only to prevent cross contamination. The buffers should all be filtered and degassed (approximately 10 min at 300 mbar) and thioglycerol added just before use. The flow rate used, except during loading the extract on the column, is 1 ml/min.

1. Wash the column with ddH_2O for at least 5 min to get rid of the ethanol.
2. Charge the column with 0.5 ml of 0.1 M $NiSO_4$ solution (*see* **Note 5**).
3. Wash the column with ddH_2O for at least 5 min.
4. Wash the column with buffer A for 10 min.
5. Wash the column with buffer B for 10 min.

6. Equilibrate the column with buffer A for 10 min.
7. Load the filtered supernatant of the ultra-centrifugation step (described in **Section 3.2.1**) at a flow rate of 0.5 ml/min.
8. Wash the column for 45 min with buffer A containing 0.5% Triton-X-100
9. Wash the column for 15 min with buffer A
10. Wash the column with 31% buffer B for 20–35 min (*see* **Note 6**).
11. Elute the protein with 90% of buffer B for 15 min (*see* **Note 7**).
12. The column is treated for 20 min with buffer B, subsequently with ddH_2O and then with 20% of ethanol for storage.
13. Equilibrate a PD-10 column with 25 ml of buffer C. Then apply 2.5 ml of the fractions containing the target protein. Elute the proteins with 3.5 ml of buffer C. Then adjust the volume of the eluate to 10 ml.

3.2.3. Calmodulin Affinity Chromatography

1. Carefully resuspend the Calmodulin (CaM) affinity resin and fill 2 ml in a PolyPrep chromatography column for gravity flow. This yields 1 ml of bed volume. Drain the liquid.
2. Equilibrate the column with at least 10 ml of buffer C.
3. Apply the diluted eluate of the PD-10 column.
4. Wash the column with 10 ml of buffer C plus 0.5% Triton-X-100.
5. Wash the column with 10 ml of buffer C.
6. Elute the proteins in five 1-ml aliquots with buffer D (*see* **Note 8**). For this purpose it is best to put the column on a 1.5-ml reaction tube and add 1 ml. Wait until the column has run dry, exchange the tube, and repeat the procedure. The fractions containing the protein are usually mainly the first three with the highest concentration in the second one. They can easily be identified with a Bradford protein assay (micro method) with 30 µl of the fractions.
7. Equilibrate a PD-10 column with buffer E and exchange the buffer of the protein containing fractions to buffer E as described in step 12 of **Section 3.2.2**.
8. Rinse a prepared dialysis tubing and pipette the eluate of the PD-10 column into it. Close the tubing using suitable clips and put it on a petri dish filled with sucrose. Also, add sucrose on top of the tubing. Incubate at 4°C for at least 2.5 h. Exchange the sucrose from time to time. When the volume has been reduced to 300–600 µl, pipette it into a 1.5-ml reaction tube. Mix this concentrated protein solution carefully but thoroughly with the same volume of 100% glycerol

and store at –20°C. Also, prepare a buffer used as a negative control in the strand displacement assay and for dilutions of the finalized enzyme preparation by mixing 500 µl of buffer E with 500 µl of glycerol and store at –20°C.

9. Regenerate the CaM column by washing with at least 10 ml of the following buffers each: buffer F, G, H, I, C. Then equilibrate the CaM column in buffer C containing 20% of ethanol for storage at 4°C. The PD-10 columns are stored in ddH_2O with sodium azide.

The purification procedure has to be performed the same way for the helicase of interest and with the helicase with the amino acid substitution introduced in the helicase motif I. The concentration of the two preparations needs to be checked, best via Coomassie staining of an SDS-Gel with BSA as a standard. This information is important to ensure the utilization of the same amount of protein (and therefore also possible contaminants) in the activity assay. Once the purification method is well established, several subsequent purifications with the same protein can be performed and the final preparations can be pooled. Then small aliquots can be made and stored at –80°C until usage. It is best to repeat the determination of the concentration of the pooled fractions after aliquoting.

3.3. Preparation of the Radioactively Labeled DNA Substrate

Make sure to comply with the law when working with radioactivity. All steps should be performed behind plexiglas shielding without lead, consisting of a thickness of at least 0.8 cm, to insulate the ß-particles from P-32. It is best to use filter tips for the pipette to prevent contamination.

In case you use [γ-^{32}P]ATP with a specific activity of 3000 Ci/mmol and 10 mCi/ml, this corresponds on the reference date to a total concentration of 3.3 pmol ATP/µl made up of approximately 1 pmol/µl [γ-^{32}P]ATP and 2.3 pmol/µl ATP without [γ-^{32}P]-label.

1. Pipette the appropriate volume of ddH_2O, 5 µl of 10 × PNK buffer, and 15 pmol of the oligonucleotide (e.g., 15 µl of a 1:100 dilution of a 100 pmol/µl stock) into a 1.5-ml reaction tube. Then add 2 µl of the T4 PNK and subsequently, with shielding, 20 pmol of ATP. On the reference date this corresponds to approximately 6 µl. Mix well and incubate at 37°C for 1 h.
2. Heat-inactivate the T4 PNK for 20 min at 67°C, then let the sample cool down to room temperature and pulse down.
3. Prepare a G-25 spin column to remove the ATP excess. First mix the bead material of the column. Then break it open and slightly unscrew. Centrifuge for 1 min according to the manual; with our special centrifuge which allowing centrifugation

behind the plexiglas screen from Neolab we centrifuge at 4000 rpm. Then put the column into a new tube, unscrew, and immediately pipette the labeling mixture on it. Close the column gently and centrifuge again. We centrifuge twice for 1 min at 4000 rpm. Discard the column.

4. Mix the eluate well and determine the volume with the help of a pipette. Then pipette 1 µl of the eluate to 4 ml of scintillation cocktail in a scintillation vial together with the tip. Vortex the scintillation vial and let it sit for at least 20 min. Then measure the activity via liquid scintillation counting. Use the direct DPM mode and measure twice for 10 min to ensure a correct value. The specific activity can be calculated, assuming that 100% of the oligonucleotide left the spin column, by the following formula: (activity/µl × determined volume of the eluate)/15 pmol.
5. To this tube add 7.5 pmol of M13mp18 single-stranded DNA and T4 PNK buffer to obtain a 1 × concentration. In case you use M13 mp18 DNA from New England Biolabs with a concentration of 250 µg/ml, this corresponds to 71 and 13.5 µl of buffer. Incubate at 95°C for 5 min then turn off the heating block and let the mixture cool down slowly (approximately 3 h) to room temperature. Then spin for a short time to assemble the whole content on the bottom of the tube.
6. Perform a gel filtration with Sephacryl S-400 spin columns to remove the excess of the labeled oligonucleotide as described in step 3 of this subsection. Do not pipette more than 50 µl on each column. Determine the activity of the eluate as described in step 4 of this subsection (*see* **Note 9**).
7. The concentration of the substrate can be calculated dividing the activity/µl (determined in step 6) by the specific activity (determined in step 4). Calculate which volume corresponds to 3 fmol of substrate (also *see* **Note 10**).

The success of the purification can be verified by mixing 1 µl of the eluate with stop solution and running a native 12% TBE-PAGE-Gel as described in the following subsection. For comparison also load the annealed substrate before purification, or heat denature 1 µl of the substrate with stop solution by heating 5 min at 95°C before shock cooling on ice.

3.4. Detection of the Helicase Activity via the Strand Displacement Assay

1. Pour two 12% TBE-PAGE gels in the Whatman Multigel-Long system. For this purpose assemble the glass plates and the insulation with the help of clamps and mix a solution of 16 ml ddH_2O, 3.2 ml 10 × TBE buffer, 12.8 ml of acrylamide (30% T, 2.67% C), 15 µl of TEMED, and 224 µl of 10% APS that is poured subsequently between the glass plates, then insert the comb. It is best to use a 16-teethed comb.

Once the gels are polymerized, remove the insulation and install them in the running chamber with 1 × TBE buffer. Prevent air bubbles trapped between the glass plates. Cool the chamber to 4°C.

2. Dilute your enzymatic preparations with the dilution buffer prepared in step 8 of **Section 3.2.3**, yielding equal (maximal) concentrations for the presumably active helicase and the helicase with amino acid substitution. Further dilute the presumably active helicase preparation by factors 2 and 4. Prepare at least 7 µl of each dilution.
3. Prepare two mastermixes (MM), one with ATP and one in which the ATP is omitted and replaced by ddH_2O for 18 reactions each. The volumes/amounts for one reaction are the following: 4 µl of reaction buffer (5 ×), 1 µl of ATP (20 ×), 1 µl of DTT (20 ×), 1 µl of BSA (20 ×), 1 µl of $MgCl_2$ (20 ×), 3 fmol of DNA substrate, ddH_2O up to 19 µl. Pipette in the following order: water, buffer, everything except substrate, and with shielding add the substrate and mix very well. Prepare one PCR stripe with five wells and pipette 66.5 µl of the MM with ATP in it and repeat the same for the MM without ATP (*see* **Fig. 14.2**).
4. Pipette 1 µl of each enzyme dilution and the dilution buffer twice in triplicates in a PCR-plate pre-cooled to –20°C on a PCR chiller (*see* **Fig. 14.2**).

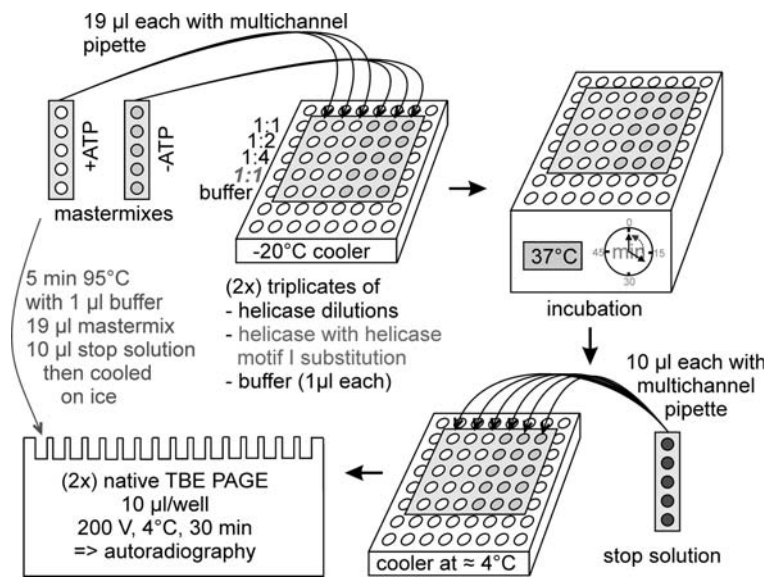

Fig. 14.2. Schematic drawing of the procedure for carrying out the strand displacement assay.

5. Bring the substrate and the enzyme together by adding 19 µl of the respective mastermix to the enzyme on a PCR chiller and mix well with a multichannel pipette.
6. Start the incubation by putting the plate on a 37°C block and incubate for 20 min. In the meantime, pipette 1 µl of dilution buffer and 19 µl of the respective leftover mastermix as well as 10 µl of stop solution into two 1.5-ml reaction tubes. Place the tubes in a block pre-warmed to 95°C for 5 min. Then place the tubes on ice immediately. Pulse down after cool-down. Now the heat denatured samples are ready for analysis on the gel.
7. Once the incubation time is over, put the reaction plate on a PCR chiller of about 4°C. Add 10 µl of stop solution with the multichannel plate in the same order as used for starting the reaction and mix well. When this step is over, place the plate at room temperature.
8. Load 10 µl into each well of the pre-cooled gel and run at 200 V for 30 min (*see* **Note 11**).
9. Remove the gels from the electrophoresis chamber. Take off the upper glass plate. Wrap the gel in plastic foil to prevent leakage of radioactivity (*see* **Note 12**).
10. Place the gel in the Instant Imager and monitor the appearance of the bands. Wait long enough to be able to detect potential minor bands indicative of nucleolytic degradation.

4. Notes

1. It may be possible to start the overnight culture from a glycerol stock (however we have experienced cases of unstable plasmid in the procedure described here for some constructs). Prepare the glycerol stock by cultivating the transformed bacteria in LB medium containing 0.1% or 1% of glucose at 37°C until an OD at 600 nm of approximately 0.3–0.6 is reached. Then add glycerol to a final percentage of 20%, mix, make aliquots and shock freeze in liquid nitrogen. The glycerol stocks are kept at –80°C. Inoculate the overnight culture with 0.1% of the glycerol stock.
2. Several lines of evidence have indicated that the addition of antibiotics is not favorable if the method is performed as described. Either the same amount or even less target protein was obtained when antibiotics were added.
3. The optimal incubation temperature and time have to be determined experimentally for each protein of interest. For the approach presented here, it is important to increase the

concentration of the target protein in the soluble fraction. Therefore, in order to investigate the best conditions for expression, the soluble protein fraction has to be prepared as described in **Section 3.2.1**. Usually the RecQ helicases are not very well expressed in the soluble fraction but accumulate in inclusion bodies. Therefore, a Coomassie stained gel is generally not sufficient and a Western Blot has to be performed. In order to conclude correctly about proteolytic degradation or incomplete translation, one should also analyze the soluble protein fraction of *E. coli* cells transformed with the expression vector without the target ORF (sometimes called "mock control"). Bands visible in this control are proteins that are detected "naturally" with the antibodies. We usually increase the incubation time when decreasing the incubation temperature and use temperatures of 28°C, 21°C, or 16°C. We could also show that sometimes the addition of 1% glucose to the LB medium greatly improves the yield of the target protein in the soluble fraction. This is sometimes not correlated to the repression of the target protein expression when not induced by IPTG, which is the usual way to explain this phenomenon (18, 19). For the addition of glucose we did not see differences for either autoclaving the LB medium with the glucose or adding the glucose afterwards. The glucose solutions added were either sterilized by autoclaving or by filtration.

4. This step removes cell debris that was not removed by the centrifugation step. The filter, composed of a prefilter of fiber glass and then a 0.45-μm polyester filter, has proved to be useful because otherwise the filters clog fast.
5. For repeated use of the same column the initial conditions can be re-established by stripping off the Ni^{2+} and recharging the column with Ni^{2+}. In practice after the washing step detailed in 1, the Ni^{2+} is stripped of for approximately 15 min with stripping buffer (20 mM NaH_2PO_4, 50 mM EDTA, pH 7.2). Afterwards the column is washed with water until the conductance is again at the water level to make sure that all EDTA is washed out. Then step 2 is performed.
6. 31% of buffer B corresponds to the optimal concentration of imidazole, which does not elute significant amounts of AtRECQ2 but as many *E. coli* proteins as possible that have bound to the column with less affinity. This imidazole concentration will have to be determined for each target protein. For this purpose perform all steps up to step 9 and then run a linear gradient of buffer B instead of the two step elution protocol. Calculate the optimal concentration of imidazole by analyzing the elution profile on a Coomassie stained SDS-PAGE gel and taking into account the volumes of the tubing.

The purification success is greater this way than via washing with a gradient, where the more suitable imidazole concentration is applied just in a very limited volume and often superimposed with the elution of the target protein.

7. The protein will be eluted in a concentrated form at the beginning; the concentration is much higher with this kind of elution than with a gradient.
8. We could show that a minimal concentration of salt, such as NaCl, is necessary for the elution of some proteins of interest. However, some contaminant proteins are eluted without NaCl. Therefore, an additional purification step can be designed on this basis.
9. If the purity after this step is not sufficient, the selectivity for the annealed substrate can be optimized by running a self-made gravity flow Sephacryl S-400 column with a height of approximately 6 cm in an empty PD-10 column with 10 mM Tris–HCl, pH 7.5, 100 mM NaCl buffer.
10. It is possible to use the prepared substrate for a longer period of time if you adjust the volume needed. In this respect it is not important to always use the same amount of radioactivity but the same amount of substrate, which includes both labeled and unlabeled substrate molecules. Over time the concentration of the labeled substrate will decrease following the exponential law with a half life of 14.3 days, but the concentration of unlabeled substrate will not (assuming that the radioactive decay only destroys the molecule in which the decay was taking place). This has to be considered in the calculation.
11. The M13mp18 DNA will stay close to the wells, whereas the separated oligonucleotide behaves in a similar way as the bromophenol blue. The optimal separation length should provide information on the appearance of products smaller than the oligonucleotide due to nucleases possibly co-purified with the helicases.
12. The resolution of the image improves, if the gel is dried before imaging. For this purpose pre-wet two cellophane sheets in ddH_2O and mantle the gel. Put filter papers on the bottom and the top and place the sandwich on a gel dryer for at least 1 h at 80°C with 300 mbar. Then remove the filter papers and wrap the gel mantled with the cellophane sheets in plastic wrap. This is necessary as the gel is not completely dry after the incubation and additional unequal drying will cause the gel to form cracks. The cellophane efficiently keeps the radioactive label in the sandwich.

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