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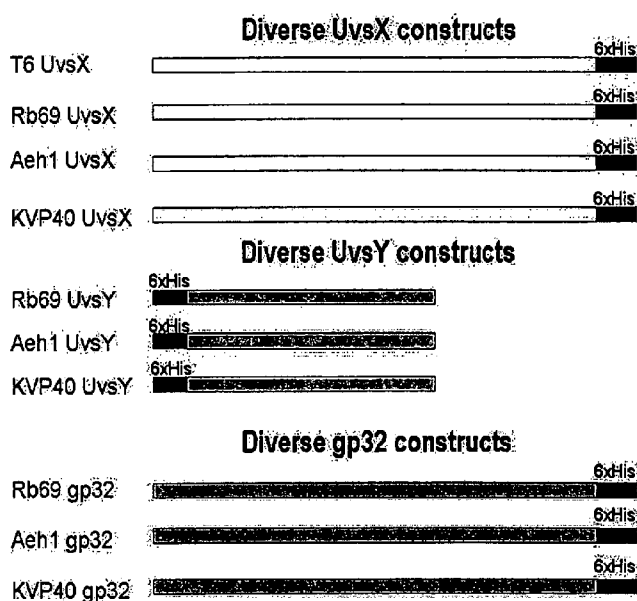
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(57) Abstract: The present invention features novel, diverse, hybrid and engineered recombinase enzymes, and the utility of such proteins with associated recombination factors for carrying out DNA amplification assays. The present invention also features different recombinase 'systems' having distinct biochemical activities in DNA amplification assays, and differing requirements for loading factors, single-stranded DNA binding proteins (SSBs), and the quantity of crowding agent employed.

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RECOMBINASE POLYMERASE AMPLIFICATION

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/798,060, filed May 4, 2006, the content of which are hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to novel hybrid and engineered recombinase enzymes, and the use of such enzymes for the amplification of nucleic acids. More specifically, the present invention relates to the use of T6, Rb69, Aeh1, and KVP40 hybrid and engineered proteins, and the use of such proteins in recombinase polymerase amplification assays.

BACKGROUND

Recombinase Polymerase Amplification (RPA) is a process in which recombinase-mediated targeting of oligonucleotides to DNA targets is coupled to DNA synthesis by a polymerase (Armes and Stemple, US application 10/371,641). RPA depends upon components of the cellular DNA replication and repair machinery. The notion of employing some of this machinery for *in vitro* DNA amplification has existed for some time (Zarling et al. US patent 5,223,414), however the concept has not transformed to a working technology until recently as, despite a long history of research in the area of recombinase function involving principally the *E.coli* RecA protein, *in vitro* conditions permitting sensitive amplification of DNA have only recently been determined (Piepenburg et al. US patent application 10/931,916, also Piepenburg et al., PlosBiology 2006). Development of a 'dynamic' recombination environment having adequate rates of both recombinase loading and unloading that maintains high levels of recombination activity for over an hour in the presence of polymerase activity proved technically challenging and needed specific crowding agents, notably PEG molecules of high molecular weight (Carbowax 20M molecular weight 15-20,000, and others described herein, particularly PEG molecular weight 35,000), in combination with the use of recombinase-loading factors, specific strand-displacing polymerases and a robust energy regeneration system.

The RPA technology depended critically on the empirical finding that high molecular weight polyethylene glycol species (particularly >10,000 Daltons or more) very profoundly influenced the reaction behaviour. It has previously been discovered that polyethylene glycol species ranging in size from at least molecular weight 12,000 to 100,000 stimulate RPA reactions strongly. While it is unclear how crowding agents influence processes within an

amplification reaction, a large variety of biochemical consequences are attributed to crowding agents and are probably key to their influence on RPA reactions.

Crowding agents have been reported to enhance the interaction of polymerase enzymes with DNA (Zimmerman and Harrison, 1987), to improve the activity of
5 polymerases (Chan E.W. et al., 1980), to influence the kinetics of RecA binding to DNA in the presence of SSB (Lavery and Kowalczykowski, 1992). Crowding agents are reported to have marked influence on systems in which co-operative binding of monomers is known to occur such as during rod and filament formation (Rivas et al., 2003) by increasing association constants by potentially several orders of magnitude (see Minton, 2001). In the RPA system
10 multiple components rely on co-operative binding to nucleic acids, including the formation of SSB filaments, recombinase filaments, and possibly the condensation of loading agents such as UvsY. Crowding agents are also well known to enhance the hybridization of nucleic acids (Amasino, 1986), and this is a process that is also necessary within RPA reactions. Finally, and not least, PEG is known to drive the condensation of DNA molecules in which they
15 change from elongated structures to compact globular or toroidal forms, thus mimicking structures more common in many *in vivo* contexts (see Lerman, 1971; also see Vasilevskaya et al., 1995; also see Zinchenko and Anatoly, 2005) and also to affect the supercoiling free energy of DNA (Naimushin et al., 2001).

Without intending to be bound by theory, it is likely that crowding agents influence
20 the kinetics of multiple protein-protein, protein-nucleic acid, and nucleic acid-nucleic acid interactions within the reaction. The dependence on large molecular weight crowding agents for the most substantial reaction improvement (probably greater than about 10,000 Daltons in size) may reflect a need to restrict the crowding effect to reaction components over a certain size (for example oligonucleotides, oligonucleotide:protein filaments, duplex products,
25 protein components) while permitting efficient diffusion of others (say nucleotides, smaller peptides such as UvsY). Further, it may also be that the high molecular weight preference might reflect findings elsewhere that as PEG molecular weight increases the concentration of metal ions required to promote DNA condensation decreases. In any case it is an empirical finding that RPA is made effective by the use of high molecular weight polyethylene glycols.

30 In addition to a need for specific type of 'crowded' reaction conditions as described above (reaction in the presence of crowding agents), effective RPA reaction kinetics depend on a high degree of 'dynamic' activity within the reaction with respect to recombinase-DNA

interactions. In other words, the available data which includes (i) reaction inhibition by ATP- γ -S, or removal of the acidic C terminus of RecA or UvsX, and (ii) inhibition by excessive ATP (Piepenburg et al., 2006) suggest that not only is it important that recombinase filaments can be formed rapidly, but also important that they can disassemble quickly. This data is
5 consistent with predictions made in earlier US patent application 10/371641. Rapid filament formation ensures that at any given moment there will be a high steady state level of functional recombinase-DNA filaments, while rapid disassembly ensures that completed strand exchange complexes can be accessed by polymerases.

Other processes must be adequately supported in the reaction environment in addition
10 to highly dynamic recombinase loading/unloading. For the benefit of later discussions there now follows a more complete list of factors to note when considering how RPA reaction may be affected by changes in activity/properties of the components:

1. As stated above there must be a high overall level of active, correctly loaded, recombinase-DNA filaments at any given moment to ensure rapid kinetics of invasion and
15 strand exchange. This is required to drive rapid reaction kinetics at low target numbers early in the reaction, as predicted by standard bi-molecular reaction kinetics, as well as to ensure non-limiting quantities of active filaments late in the reaction when targets become highly abundant and could easily out-titrate the loaded filaments.

2. Filaments must be dynamic, capable of rapid disassembly as well as assembly, to
20 ensure that strand exchange processes work rapidly, and to avoid filament 'lock-up' in unproductive protein-DNA conformations (should they arise).

3. Recombinases should have a strong preference for single-stranded DNA, and a relatively weaker preference for double-stranded DNA. This ensures the correct partitioning of recombinase onto the oligonucleotides, and is very important in the late phase of the
25 reaction when significant quantities of duplex DNA accumulate. This duplex DNA may otherwise compete too effectively for recombinase and slow the reaction too rapidly. A difference in disassembly rates on duplex DNA would also enhance factor (ii) insofar as accelerating disassembly of productive exchange complexes. Observations consistent with 'out-titration' activity of excess duplex DNA, such as decreases in reaction rate late in the
30 reaction, or if excess DNA is present early in the reaction, have been made.

4. Hybridization of single-stranded DNA's to one another must be supported under any given reaction condition. RPA has the potential to generate single-stranded DNA

products which may only be converted to new duplex targets following hybridization of the complementary priming oligonucleotide to initiate DNA synthesis. As saturating quantities of single-stranded DNA binding proteins (i.e. loading proteins, single-stranded DNA binding proteins and recombinases) are present in the reaction environment, these hybridization processes must be supported/aided by these proteins. SSB's and recombinases have some melting/hybridization activities on duplex/single-stranded DNA's, and probably demonstrate differential levels of melting/hybridization activity. Thus the relative proportions of recombinase and SSB of loading may influence the rate behaviour for hybridization, and this may also depend on the species of SSB and recombinase employed. If either the SSB or recombinase does not, or only poorly, supports hybridization of single-stranded DNAs to one another, then the reaction may be compromised.

5. The temporal change in reaction composition with regard to pH, anion accumulation, generation of ADP, of AMP, pyrophosphate, and other nucleotide species may be strongly influenced by the recombinase employed. Furthermore recombinases may respond differentially to the ionic and pH environment. Rates of nucleotide hydrolysis affect the accumulation of the afore-mentioned species, and their accumulation may in turn influence the activity in the reaction of recombinases and polymerases. For example accumulation of phosphate and pyrophosphate may inhibit recombinase processes, while the accumulation of ADP (and possibly AMP) can affect DNA on-off kinetics of the recombinase. Notably bacteriophage T4 UvsX protein has been reported to hydrolyse ATP to both ADP and AMP, a property not attributed to other recombinases to date. Recombinases may also hydrolyse dATP, UTP and potentially other nucleotides. Different nucleotides may affect the DNA binding stabilities of complexes on ssDNA and dsDNA, for example dATP has been noted to increase the stability of RecA on ssDNA. Without intending to be bound by theory, the particular properties of a recombinase with respect to its DNA binding domains and nucleotide binding/catalysis domains may have significant impact on reaction rate and effectiveness in generating strong signals late in the reaction.

30 *Previously Established RPA conditions.*

Effective RPA reactions have previously been demonstrated using both *E.coli* RecA (in a heterologous system with compromised gp32 protein) and with the T4 phage UvsX

protein (when combined with the T4 phage UvsY protein) (Piepenburg et al., 2006). In both cases the employment of polyethylene glycol was found to be absolutely necessary for amplification to occur with any useful efficiency when templates were present at concentrations below roughly nanomolar levels (or roughly below the order of about 10^{10} target molecules per microliter).

Experimentation showed the importance of PEG in stimulating secondary, tertiary and yet further invasion events when using oligonucleotides directed towards the ends of linear templates, said oligonucleotide initially having a 5' overhang relative to the initial target, but being flush to later targets due to the activity of 'backfire' synthesis (Piepenburg et al. U.S.S.N. 10/931,916). Fully embedded targets proved to be even more intractable, almost certainly due to the topological constraints associated with the recombination products caused by the outgoing strand being wound unfavourably around the newly formed duplex. Without intending to be bound by any theory, the huge increase in efficiency of initiating replication from these more unstable intermediates in the presence of PEG may depend on stability conferred by the crowding agent on the complexes, on altered DNA conformation and coiling (such as DNA condensation), on much higher association constants for the polymerase gaining access to the intermediates, and/or a very great increase in the frequency of recombination events leading to more 'chances' of the polymerase grabbing the intermediate and elongating.

An RPA system utilizing bacteriophage T4 UvsX, T4 UvsY, and T4gp32, a *B.subtilis* PolII large fragment, and PEG compound (carbowax 20M) is effective for amplifying duplex DNA sequences up to about 1 kilobase in length (Piepenburg et al., 2006). Average doubling times of as little as 40 seconds or less have been attained for fragments of roughly 300 nucleotides, and DNA accumulates to levels useful for detection by a variety of means, even when targets are initially present at levels below 10 copies. Despite this robust behaviour there exists a need for the identification of other recombinases, their associated loading components and single stranded DNA binding proteins, due to the strict necessity for very rapid kinetics and strong signals for the implementation of the RPA system in commercially useful products. The present invention meets these needs and other needs.

SUMMARY OF THE INVENTION

This disclosure provides enabling data on the use of alternative recombinase/accessory factor systems for performing RPA reactions. As evidenced herein, bacteriophage T6 UvsX, bacteriophage Rb69 UvsX, UvsY and gp32, and bacteriophage Aeh1 UvsX, UvsY, and gp32 can be employed successfully in RPA reactions. Additionally, evidence that bacteriophage KVP40 UvsX and UvsY may also be able to support RPA reactions is included, although problems were encountered in the production of KVP40 gp32 that limited this analysis. In general it was discovered that variation in the concentration of reactants must be performed to identify optimal conditions for each system, and there are observable differences in overall kinetic activity. The present invention provides evidence of limited cross-compatibility between reaction components generated from different species. In general the requirement for co-employment of UvsX and UvsY from the same or similar species was observed, while gp32 may be less stringently matched. Also provided herein are mutant and chimeric recombinase proteins, in particular the use of altered T6 and Rb69 UvsX proteins, and chimeric T4 and Rb69 UvsY proteins, and the analysis thereof. This analysis leads to identification of residues influencing the assayable behaviour of the proteins in RPA reactions. As provided herein, some, but not all, of the character of the T4 UvsX protein derives from a unique serine residue within the Walker A motif. Without intending to be bound by any theory, the resulting re-iteration of a lysine-serine dipeptide within the motif may underpin the hydrolysis of ATP to both ADP and AMP by this protein. Modification of T6 UvsX protein to contain this re-iteration results in altered (improved) RPA activity when monitored in real-time. Such modified UvsX demonstrates changed reaction kinetics when assayed by proprietary fluorescent probes, in particular exhibiting steeper fluorescent signal-generation curves during the late phase of the amplification reaction. Also provided herein is the discovery that regions of myoviridae UvsX proteins which are predicted to be equivalents to DNA binding loop 2 of *E.coli* are variable and impart distinctive activities UvsX hybrids used in RPA reactions. Rb69 UvsX is an unusual UvsX molecule in regard to this sequence, more closely resembling the bacterial homologs. The present invention provides a model for structure/sequence compatibility in the surface region of recombinase enzymes that binds both nucleic acids and ATP, and how this evidence may be employed to 'tune' and improve (alter) recombinase activity. Surprisingly it was discovered that T6 UvsX, in particular, can function moderately well with a complete absence of UvsY protein. This property may be evident for other UvsX species although less markedly. Finally the present invention provides

the use of manganese ions to support RPA reactions, the use of heparin to improve signal:noise ratios, the use *S.aureus* Pol I as the polymerase employed in RPA reactions, and *E.coli* exonuclease III to process and unblock primer ends in some cases to permit elongation.

The first RPA embodiment of the invention is directed to a process (method) of
5 recombinase polymerase amplification of a double stranded target nucleic acid molecule. In the first step of the process, a first and a second single stranded nucleic acid primer is contacted with a recombinase (e.g., UvsX), a recombinase loading agent (e.g., UvsY) and a single strand DNA binding protein (e.g., gp32) to form a first and a second nucleoprotein
10 primer. The single stranded nucleic acid primers are specific for and are complementary to the target nucleic acid molecule. In this case each of the recombinase (e.g., UvsX), recombinase loading agent (e.g., UvsY) and single strand DNA binding protein (e.g., gp32) are derived from a myoviridae phage. Further, no more than two of the recombinase (e.g., UvsX), recombinase loading agent (e.g., UvsY) and single strand DNA binding protein (e.g., gp32) are T4 phage proteins.

15 In the second step, the first nucleoprotein primer is contacted to the double stranded target nucleic acid molecule to create a first D loop structure at a first portion of the double stranded target nucleic acid molecule (Step 2a). Further, the second nucleoprotein primer is contacted to the double stranded target nucleic acid molecule to create a second D loop structure at a second portion of the double stranded target nucleic acid molecule (Step 2b).
20 The D loop structures are formed such that the 3' ends of the first nucleic acid primer and said second nucleic acid primer are oriented toward each other on the same double stranded target nucleic acid molecule without completely denaturing the target nucleic acid molecule. It should be noted that step 2a and step 2b can be performed in any order or simultaneously.

In a D loop structure, the primer is hybridized to one strand of the double stranded
25 target nucleic acid molecule to form a double stranded structure. The second strand of the target nucleic acid molecule is displaced by the primer. The structure resembles a capital D where the straight part of the D represents the double stranded part of the structure and the curved part of the D represents the single stranded displaced second strand of the target nucleic acid.

30 In the third step, the 3' end of the first and the second nucleoprotein primer is extended with one or more polymerases capable of strand displacement synthesis and dNTPs to generate a first and second double stranded target nucleic acid molecule and a first and

second displaced strand of nucleic acid. The first and second double stranded target nucleic acid molecules may serve as target nucleic acid molecules in step two during subsequent rounds of amplification.

Steps two and step 3 are repeated until a desired degree of amplification of the target nucleic acid is achieved. A desired degree of amplification may be at least 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 , or 10^9 fold amplification.

During the amplification process described above, the first and second displaced strand of nucleic acid may hybridize to each other after step (c) to form a third double stranded target nucleic acid molecule.

10 In any of the processes of this disclosure, the recombinase (e.g., UvsX), recombinase loading agent (e.g., UvsY) and single strand DNA binding protein (e.g., gp32) may be derived from a myoviridae phage. The myoviridae phage may be, for example, T4, T2, T6, Rb69, Aeh1, KVP40, Acinetobacter phage 133, Aeromonas phage 65, cyanophage P-SSM2, cyanophage PSSM4, cyanophage S-PM2, Rb14, Rb32, Aeromonas phage 25, Vibrio phage
15 nt-1, phi-1, Rb16, Rb43, Phage 31, phage 44RR2.8t, Rb49, phage Rb3, or phage LZ2. In a preferred embodiment, the combination of Rb69 UvsX, Rb69 UvsY and Rb69 gp32 may be used. In another preferred embodiment, the combination of Aeh1 UvsX, Aeh1 UvsY and Rb69 gp32 may be used. In another preferred embodiment, the combination of T4 UvsX, T4 UvsY and Rb69 gp32 may be used. In another preferred embodiment, the combination of T4
20 UvsX, Rb69 UvsY and T4 gp32 may be used.

Further, in any of the processes of this disclosure, the recombinase (e.g., UvsX), recombinase loading agent (e.g., UvsY) and single strand DNA binding protein (e.g., gp32) can each be native, hybrid or mutant proteins from the same or different myoviridae phage sources. A native protein may be a wildtype or natural variant of a protein. A mutant protein
25 (also called a genetically engineered protein) is a native protein with natural or manmade mutations such as insertions, deletions, substitutions, or a combination thereof, that are at the N terminus, C terminus, or interior (between the N terminus and the C terminus). A hybrid protein (also called a chimeric protein) comprises sequences from at least two different organisms. For example, a hybrid UvsX protein may contain an amino acid from one species
30 (e.g., T4) but a DNA binding loop from another species (e.g., T6). The hybrid protein may contain improved characteristics compared to a native protein. The improved characteristics

may be increased or more rapid RPA amplification rate or a decreased or more controllable RPA amplification rate.

In any process of this disclosure, the recombinase (e.g., UvsX) may be a mutant UvsX. In a preferred embodiment, the mutant UvsX is an Rb69 UvsX comprising at least one mutation in the Rb69 UvsX amino acid sequence, wherein the mutation is selected from the group consisting of (a) an amino acid which is not histidine at position 64, a serine at position 64, the addition of one or more glutamic acid residues at the C-terminus, the addition of one or more aspartic acid residues at the C-terminus, and a combination thereof. In another preferred embodiment, the mutant UvsX is a T6 UvsX having at least one mutation in the T6 UvsX amino acid sequence, wherein the mutation is selected from the group consisting of (a) an amino acid which is not histidine at position 66; (b) a serine at position 66; (c) the addition of one or more glutamic acid residues at the C-terminus; (d) the addition of one or more aspartic acid residues at the C-terminus; and (e) a combination thereof.

In any process of this disclosure where a hybrid protein is used, the hybrid protein may be a UvsX protein comprising at least one region which comprises an amino acid sequence from a different UvsX species. The region may be, for example, the DNA-binding loop-2 region of UvsX.

Any of the RPA process of this disclosure may be performed in the presence of a crowding agent. The crowding agent may be selected from the group comprising polyethylene glycol, polyethylene oxide, polyvinyl alcohol, polystyrene, Ficoll, dextran, PVP, albumin. In a preferred embodiment, the crowding agent has a molecular weight of less than 200,000 daltons. Further, the crowding agent may be present in an amount of about 0.5% to about 15% weight to volume (w/v).

Any of the RPA processes of this disclosure may be performed with a polymerase which is a large fragment polymerase. The large fragment polymerase may be selected from the group consisting of *E. coli* Pol I, *Bacillus subtilis* Pol I, *Staphylococcus aureus* Pol I, and homologues thereof.

Any of the RPA processes of this disclosure may be performed in the presence of heparin. Heparin may serve as an agent to reduce the level of non-specific primer noise, and to increase the ability of *E. coli* exonuclease III or *E. coli* exonuclease IV to rapidly polish 3' blocking groups or terminal residues from recombination intermediates.

Further, any of the RPA processes of this disclosure may be performed with a blocked primer. A blocked primer is a primer which does not allow elongation with a polymerase. Where a blocked primer is used, an unblocking agent is also used to unblock the primer to allow elongation. The unblocking agent may be an endonuclease or exonuclease which can
5 cleave the blocking group from the primer. Preferred unblocking agents include E.coli exonuclease III and E.coli endonuclease IV.

Any of the RPA processes of this disclosure may be performed in the presence of about 1 mM to about 3 mM divalent manganese ions. In a preferred embodiment, the manganese ions replace the magnesium ions and the reaction may be performed with or
10 without magnesium.

Furthermore, UvsY may be optionally omitted from any of the RPA reactions of this disclosure. That is, any of the RPA reactions of this disclosure may be performed in the absence of UvsY.

The second RPA embodiment of the invention is directed to a process (method) of
15 recombinase polymerase amplification of a double stranded target nucleic acid molecule. In the first step of the process, recombinase (e.g., UvsX), recombinase loading agent (e.g., UvsY) and single strand DNA binding protein (e.g., gp32) are contacted with a first single stranded nucleic acid primer specific for the double stranded target nucleic acid molecule to form a population of first nucleoprotein primer, wherein the recombinase (e.g., UvsX),
20 recombinase loading agent (e.g., UvsY) and single strand DNA binding protein (e.g., gp32) are each derived from a myoviridae phage, and wherein no more than two of the recombinase (e.g., UvsX), recombinase loading agent (e.g., UvsY) and single strand DNA binding protein (e.g., gp32) are T4 phage proteins.

In the second step, the first nucleoprotein primer is contacted with the double stranded
25 target nucleic acid molecule to form a first D loop structure at a first portion of said double stranded target nucleic acid molecule without completely denaturing the target nucleic acid molecule;

In the third step, the 3' end of the first nucleoprotein primer is extended with one or more polymerases capable of strand displacement synthesis and dNTPs to generate a double
30 stranded target nucleic acid molecule and a displaced strand of nucleic acid molecule;

In the fourth step, a second single stranded nucleic acid primer is hybridized to the displaced strand of nucleic acid molecule to form a hybridized second single stranded nucleic acid primer;

In the fifth step, the hybridized second single stranded nucleic acid primer is
5 elongated to generate a double stranded target nucleic acid molecule;

The second through fifth steps of the reaction is continued until a desired degree of amplification is reached.

All other aspects of this second RPA embodiment is similar to that of the first RPA embodiment including the desired degree of amplification and the choice of proteins
10 (recombinase, loading agent, single stranded DNA binding protein) etc. These parameters are described above for the first RPA embodiment. We have found, surprisingly, that RPA would function even if only one of the nucleic acid primers was coated with recombinase/recombinase loading agent/single stranded DNA binding protein. That is, an RPA may be performed with one primer which is uncoated and one primer which is coated
15 with any one or a combination of recombinase, recombinase loading agent, and single stranded DNA binding protein.

The production of a coated primer and an uncoated primer may be made in a number of methods. In one method, only one primer is contacted to any one or a combination of recombinase, recombinase loading agent, and single stranded DNA binding protein before
20 commencement of RPA. In another method, both primers are contacted to any one or a combination of recombinase, recombinase loading agent, and single stranded DNA binding protein. However, one primer is incapable of attaching sufficient protein to be able to generate a D loop on a target double stranded nucleic acid. This may be because the primer is too short or contain unusual nucleic acids such that it cannot bind sufficient protein for
25 recombination. Nevertheless, to our surprise, RPA is possible even if only one primer is capable of forming D loops. RPA is possible in this circumstance because the primer which cannot form a D loop can hybridize to any displaced strand generated from the D loop capable primer (the recombinase coated primer) to initiate DNA synthesis.

Another embodiment of the invention is directed to a mutant or hybrid Rb69 UvsX
30 protein with an amino acid sequence selected from the group consisting of (a) an amino acid which is not histidine at position 64; (b) a serine at position 64; (c) the addition of one or

more glutamic acid residues at the C-terminus; (d) the addition of one or more aspartic acid residues at the C-terminus; (e) the replacement of DNA-binding loop-2 region with a DNA-binding loop-2 region from a UvsX protein which is not Rb69 UvsX; and (f) a combination thereof. An example of such mutants or hybrids may be found, for example, in SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, SEQ ID NO:119, SEQ ID NO:120, or SEQ ID NO:121.

Another embodiment of the invention is directed to a mutant or hybrid T6 UvsX protein having at least one mutation in the amino acid sequence, wherein the mutation is selected from the group consisting of (a) an amino acid which is not histidine at position 66; (b) a serine at position 66; (c) the addition of one or more glutamic acid residues at the C-terminus; (d) the addition of one or more aspartic acid residues at the C-terminus; (e) the replacement of DNA-binding loop-2 region with a DNA-binding loop-2 region from a UvsX protein which is not T6 UvsX; (f) a valine at position 164, (g) a serine at position 166, and (h) a combination thereof. See, for example, SEQ ID NO:105 and SEQ ID NO:106.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a schematic representation of clones used to generate variant UvsX, UvsY and gp32 proteins.

Figure 2 shows a primary sequence alignment of bacteriophage T4 UvsX with *E. coli* recA. T4 UvsX sequence is: MSDLKSRLIK ASTSKLTAE TASKFFNEKD

VVRTKIPMMN IALSGEITGG MQSGLLILAG PSKSFKSNFG LTMVSSYMRQ
YDPAVCLFYD SEFGITPAYL RSMGVDPERV IHTPVQSLEQ LRIDMVNQLD
AIERGEKV VV FIDSLGNLAS KKETEDALNE KVVSDMTRAK TMKSLFRIVT
PYFSTKNIPC IAINHTYETQ EMFSKTVMG GTGPMYSADT VFIIGKRQIK
DGSDLQGYQF VLNVEKSRTV KEKSKFFIDV KFDGGIDPYS GLLDMALELG
FVVKPKNGWY AREFLDEETG EMIREEKSWR AKDTNCTTFW GPLFKHQFPR
DAIKRAYQLG AIDSNEIVEA EVDELINSKV EKFKSPESKS KSAADLETDL
EQLSDMEEFN E (SEQ ID NO:1). The *E. Coli* RecA sequence is as follows:
MAIDENKQKA LAAALGQIEK QFGKGSIMRL GEDRSMDVET ISTGSLSLDI
ALGAGGLPMG RIVEIYGPE SGKTTLTLQV IAAQREGKT CAFIDAEHAL
DPIYARKLGV DIDNLLCSQP DTGEQALEIC DALARSGAVD VIVVDSVAAL
TPKAEIEGEI GD SHMGLAAR MMSQAMRKLA GNLKQSNLTL IFINQIRMKI
GVMFGNPETT TGGNALKFYA SVRLDIRRIG AVKEGENVVG SETRVKVVKN

KIAAPFKQAE FQILYGEGIN FYGELVDLGV KEKLIEKAGA WYSYKGEKIG
 QGKANATAWL KDNPETAKEI EKKVRELLLS NPNSTPDFSV DDSEGVAETN EDF
 (SEQ ID NO:2).

Figure 3 shows a representative 3-D structure of a model of an active *E.coli* recA
 filament with superimposition and labelling of equivalent T4 UvsX residues based on
 primary sequence alignment. Figure 3A is a screenshot looking down the axis of the model
 RecA filament with the central hole being the approximate location of bound DNA. The
 approximate location of the Walker A motif and mobile DNA binding loops is indicated for a
 single subunit and is on the surface facing the nucleic acid. Figures 3B and 3C are two
 zoomed shots taken of the region to which ATP is bound on the surface indicated in 3A.

Figure 4 shows the primary sequence alignment of T4 and T6 gp32 and UvsY proteins.
 The T6 gp32 sequence is as follows: MFKRKSTAEL AAQMAKLGN KGGFSSSEDKG
 EWKLKLDNAG NGQAVIRFLP SKNDEQAPFA ILVNHGFKKN GKWYIETCSS
 THGDYDSCPVC CQYISKNDLY NTDNKEYSLV KRKTSYWANI LVVKDPAAPE
 NEGKVFKYRF GKKIWDKINA MIAVDVEMGE TPVDVTCWE GANFVLKVKQ
 VSGFSNYDES KFLNQSAIPN IDDESFKEL FEQMVDLSEM TSKDKFKSFE
 ELSTKFSQVM GTAAMGGAAA TAAKKADKVA DDLDAFNVD D FNTKTEDDFM
 SSSGSSSSA DDTDLDDLND L (SEQ ID NO:3). The T4 gp32 sequence is as follows:
 MFKRKSTAEL AAQMAKLGN KGGFSSSEDKG WKLKLDNAGN GQAVIRFLPS
 KNDEQAPFAI LVNHGFKKNG KWIETCSST HG DYDSCPVC QYISKNDLYN
 TDNKEYSLV KRKTSYWANIL VVKDPAAPEN EGKVFKYRFG KKIWDKINAM
 IAVDVEMGET PVDVTCWEG ANFVLKVKQV SGFSNYDESK FLNQSAIPNI
 DDESFKELF EQMVDLSEMT SKDKFKSFEE LNTKFGQVMG TAVMGGAAAT
 AAKKADKVAD DLDAFNVD D FNTKTEDDFMS SSSGSSSSAD DTDLDDLND L
 (SEQ ID NO:4). The T4 UvsY sequence is as follows: MRLEDLQEEL KKDVFIDSTK
 LQYEAANNVM LYSKWL NKHS SIKKEMLRIE AQKKVALKAR LDYYSGRGDG
 DEFSMDRYEK SEMKTVLSAD KDVLKVDTS L QYWGILLDFC SGALDAIKSR
 GFAIKHIQDM RAFEAGK (SEQ ID NO:5). The T6 UvsY sequence is as follows:
 MRLEDLQEEL KKDVFIDSTK LQYEAANNVM LYSKWL NKHS SIKKEMLRID
 AQKKVALKAK LDYYSGRGDG DEFSMDRYEK SEMKTVLSAD KDVLKVDTS L
 QYWGILLDFC SGALDAIKSR GFAIKHIQDM RAFEAGK (SEQ ID NO:6).

Figure 5 shows the primary sequence alignment of diverse UvsX proteins. The T4UvsX sequence is as follows: MSDLKSRLIK ASTSKLTAEI TASKFFNEKD VVRTKIPMMN IALSGETGG MQSGLLILAG PSKSFKSNFG LTMVSSYMRQ YPDAVCLFYD SEFGITPAYL RSMGVDPERV IHTPVQSLEQ LRIDMVNQLD

5 AIERGEKVVV FIDSLGNLAS KKETEDALNE KVVSDMTRAK TMKSLFRIVT PYFSTKNIPC IAINHTYETQ EMFSKTVMGG GTGPMYSADT VFIIGKRQIK DGSDLQGYQF VLNVEKSRTV KEKSKFFIDV KFDGGIDPYS GLLDMALELG FVVKPKNGWY AREFLDEETG EMIREEKSWR AKDTNCTTFW GPLFKHQFPR DAIKRAYQLG AIDSNEIVEA EVDELINSKV EKFKSPESKS KSAADLETDL

10 EQLSDMEEFN E (SEQ ID NO:7). The t6UvsX sequence is as follows: MSIADLKSRL IKASTSKMTA ELTTSKFFNE KDVIRTKIPM LNIAISGAID GGMQSGLTIF AGPSKHFKSN MSLTMVAAYL NKYPDAVCLF YDSEFGITPA YLRSMGVDPDE RVIHTPIQSV EQLKIDMVNQ LEAIERGEKV IVFIDSIGNM ASKKETEDAL NEKSVADMTR AKSLKSLFRI VTPYFSIKNI PCVAVNHTIE TIEMFSKTVM

15 TGGTGVMYSA DTVFIIGKRQ IKDGSDLQGY QFVLNVEKSR TVKEKSKFFI DVKFDGGIDP YSGLLDMALE LGFVVKPKNG WYAREFLDEE TGEMIREEKS WRAKDTNCTT FWGPLFKHQP FRDAIKRAYQ LGAIDSNEIV EAEVDELINS KVEKFKSPES KSKSAADLET DLEQLSDMEE FNE (SEQ ID NO:8). The Phage133UvsX sequence is as follows: MSSLKERLIK ASTSKMTAEI TSKKFFNDKT VVRTRIPMLN

20 IAISGALNGG MQSGLTIFAG PSKHFKSNMG LTMVAAYMKA FPDVCMFYD SEFGITPAYL KAMGVDPDRV IHTPVQSVEQ LKIDMTNQLE EVKRGEKVIV FIDSIGNLAS KKETEDALNE KTTADMTRAK ALKSLFRIVT PYFSIKDIPC VAVNHTLQTL EMFSKEVMTG GTGVMYSADT VFFIGKRQVK DGTLAGYEF ILKAEKSRMV KEKSVFPITV KFDGGIDPYS GLLEMATDLG FVVKPKVGWY

25 KRAMMVDGVM QHEEKSWRAK DTDSIDFWGP LFKHDEFKRA IETRYQLGSI ESDAEVDAEV DALIGSKTTA KISGVNFGPA ESAADKEQQL EDFVDED (SEQ ID NO:9). The Rb69 UvsX sequence is as follows: MSDLKSRLIK ASTSKMTADL TSKSLFNNRD EVPTRIPMLN IALGGALNAG LQSGLTIFAA PSKHFKTLFG LTMVAAYMCK YKDAICLFYD SEFGASESYF RSMGVDLDRV VHTPIQSVEQ

30 LKVDMTNQLD AIERGDKVII FIDSIGNTAS KKETEDALNE KVVGDMSRAK ALKSLFRIVT PYLTIKDIPC VAINHTAMEI GGLYPKEIMG GGTGILYSAN TVFFISKRQV KEGTELTGYD FTLKAEKSRT VKEKSTFPIT VNFDDGGIDPF

SGLLEMATEI GFVVKPKAGW YAREFLDEET GEMIREEKSW RAKATDCVEF
 WGPLFKHKPF RDAIETKYKL GAISSIKEVD DAVNDLINCK ATTKVPVKTS
 DAPSAADIEN DLDEMEDFDE (SEQ ID NO:10). The Aeh1UvsX sequence is as follows:

MAKGIKTAKT GNLGSLMSKL AGTSSNKMSS VLADSKFFND KDCVRTRVPL
 5 LNLAMSGELD GGLTPGLTVL AGPSKHFKSN LSLVFVAAYL RKYPDAVCIF
 FDNEFGSTPG YFESQGVDIS RVIHCPFKNI EELKFDIVKK LEAIERGDRV
 IVFVDSIGNA ASKKEIDDAI DEKSVSDMTR AKQIKSLTRM MTPYLTVNDI
 PAIMVAHTYD TQEMYSKKVV SGGTGITYSS DTVIIIQRQQ EKD GKELLGY
 NFVLNMEKSR FVKEQSKLPL EVTFQGGINT YSGMLDIALE VGFVVKPSNG
 10 WFSRAFLDEE TGELVEEDRK WRRADTNCLE FWKPMFAHQP FKTACSDMFK
 LKSVAVKDEV FDEVDELFSG EAEMPVNMGR KLDTADQEEI DQLEEVDVEG
 SDSDELFANL D (SEQ ID NO:11). The Ae65UvsX sequence is as follows:

MAKKAKVVNS GDLLERLNGT SSNKMSAMLA ESIFFNEKDT IRTRVPIINL
 MMSGRLDGGI TPGLTCIAGP SKHFKNLSL VMVSAYLRKY PKAVCLFFDN
 15 EFGSTPDYFT SQGVDISRVV HCPFIDVEEL KFDIVKKLES ITRGDKVIIY
 IDSIGNVASK KELQDAKDEK SAQDMTRAKQ IKSLFRMVTP YLTVLDIPCI
 AVNHTYETQE MFSKTVMSGG TGPMYSADTV IILGKQQDKD GKELLGYNFV
 MNAEKSRAIK ESKLDLMVS FEGGINTYSG LLKIAQELGF VTKPQNARYQ
 RNFLDLEPGE MVIPEDEKKW TEEESDSLEF WKPMFSHKPF MDAVSNAAYKL

20 KAVEVSQEVF DEV DQLFG (SEQ ID NO:12). The Kvp40UvsX sequence is as follows:

MSDLMKSLKK SSTSGYAQVL SESQFMFDKD HTRTYVPAIN IAFSGEVDGG
 LTSGLTVLAG PSKHFKSNLG LVGVAAYLKK YPDAVCVFID TEFGITPSYL
 RSQGVDPDRV LHIQCESVER MKFEMANQLK DLAERKRAKK AGEEPDRVIF
 FIDSVGNVAS AKEIDDAQNE KSVADMSRAK QLKSLFRIIT PYFTMLDIPC
 25 IAINHTYQTQ EIYSKTVMSG GTGIMYSADT VIILGKQQEK DGKDIIIGYHF
 IMNIEKSRFV KEKMKVPLTV TYENGIDPFS GLLDIALQTG HVVKPSNGWY
 QRATVDEETG EMIVEEKKYR AKETQTISFW KDIINSPTFK EGVKRIYCLG
 QLDESELFGE VDSLFD (SEQ ID NO:13). The Rb43UvsX sequence is as follows:

MSNKALLKKL IKNSNSQSAA ILSESDVFNN ITKTRTRVPI LNLALSGAFD
 30 GGLTSGTLTF AGPSKHFKSN LGLVTVSAYL KANEDAVCLF YDSEKGVTKS
 YLKSMGVDPD RVVYTRITTV EQLRNDVVSQ LDALERGDKV IIFVDSVGNT
 ASKKELADAL SDNDKQDMTR AKALKGMFRM VTPYLADLDI PMVCICHTYD

- TQEMYSKKVI SGGTGLMYSA.DTAILGKQQ VKEGTEVVGY DFIMNIEKSR
 FVKEKSKFPL HVTYEGGISM YSGLLDLAME MNFVQTPTKG WRGRAFLNTE
 TGELELEKK WRESETNSIE FWRPLFTHQP FLDAIQDKYR IPDKEITDGA
 ALEDLYSTDE PESNKIDLDD DIPDDIGIDQ DEEPIM (SEQ ID NO:14). The
 5 PSSM2UvsX sequence is as follows: MDFLKEIVKE IGDEYTQVAA DIQENERFID
 TGSYIFNGLV SGSIFGGVSS SRITAIAGES STGKTYFSLA VVKNFLDNNP
 DGYCLYFDTE AAVNKGLLES RGIDMNRLVV VNVVTIEEFR SKALRAVDIY
 LKTSEEERKP CMFVLDSLGM LSTEKEIRDA LDDKQVRDMT KSQLVKGAFR
 MLTLKLGQAN IPLIVTNHTY DVIGSYVPTK EMGGGSGGLKY AASTIYLSK
 10 KKEKDQKEVI GNLIKAKTHK SRLSKENKEV QIRLYYDERG LDRYYGLLEL
 GEIGGMWKNV AGRYEMNGKK IYAKEILKNP TEYFTDDIME QLDNIAKEHF
 SYGTN (SEQ ID NO:15). The PSSM4UvsX sequence is as follows: MNFLKDIAKE
 IGNDYASLVS EGVSAAGDTAG FIDTGSYIFN ALLSGSIYGG IPNNKITAIA
 GETSTGKTFF CLGMVQHFL ESNPDAGVIYF ESESAISKQM IEDRGIDSNR
 15 MLLVPVTTVQ EFRLQAIKIL DKYNEQTAE E RKPLMFVLDS LGMLSTSKEV
 EDSEAGKETR DMTRAQVVKS IFRVLTCLKG KANVPLIVTN HTYDVVGAYI
 PTKEMGGGSG LKYAASTIVY LSKKKEKNGK EVVGNIKCK TAKSRLTKEN
 SDVETRLYYD RGLDRYYGLL ELGEKHGVFS RKGNRVVVG D SSVYPSAILA
 DPDKYFTEEL MEKLDEAAAK EFRYGN (SEQ ID NO:16).
- 20 Figure 6 shows the primary sequence alignment of diverse UvsY proteins. The
 T4UvsY sequence is as follows: MRLEDLQEEL KKDV FIDSTK LQYEAANNVM
 LYSKWL NKHS SIKKEMLRIE AQKKVAL KAR LDYYSGRGDG DEFSMDRYEK
 SEMKTVLSAD KDVLKVDTSL QYWGILLDFC SGALDAIKSR GFAIKHIQDM
 RAFEAGK (SEQ ID NO:17). The T6UvsY sequence is as follows: MRLEDLQEEL
 25 KKDVFIDSTK LQYEAANNVM LYSKWL NKHS SIKKEMLRID AQKKVAL KAK
 LDYYSGRGDG DEFSMDRYEK SEMKTVLSAD KDVLKVDTSL QYWGILLDFC
 SGALDAIKSR GFAIKHIQDM RAFEAGK (SEQ ID NO:18). The Rb69UvsY sequence is
 as follows: MKLEDLQEEL DADLAIDTTK LQYETANNVK LYSKWLRKHS
 FIRKEMLRIE TQKKTALKAR LDYYSGRGDG DEFSMDRYEK SEMKTVLAAD
 30 KDVLKIETTL QYWGILLEFC SGALDAVKSR SFALKHIQDM REFEAGQ (SEQ ID
 NO:19). The phage133UvsY sequence is as follows: MTLEDLQAEL KKDVLVDMTQ
 LQTEAAENIN LYCKWSTKYS NIRKSILSLD AQRKKHTKTK LDYYSGRGDE

VSM DRYERSE MKTVLSGDAD ILTVETKIQY FTIMLEFCGN AMDAIKSRGF
 AIKNIIDLRQ FEAGK (SEQ ID NO:20). The Aeh1UvsY sequence is as follows:
 MTLDELKEEL KADLPIKLT A VQTEVAENPV LYGKWNRYLA DINREITRLD
 AERKKMLRDR FMFYTG RSED EVCMDVYSPT ELKTVIAGDE EVIKKNAAVE
 5 LSQAKADFCR QSMEAVRQRG FSLRAIIDCR KLEAGE (SEQ ID NO:21). The
 Rb43UvsY sequence is as follows: MTELKLEDLQ AELEQDMLID PLKLQSESAD
 IPKIWSKWLR YHSNAKKKLI QLQARKEADV KERLLYYTGR HETEMTDVIY
 TGSGEIKIAI NGDPKIVEVN KLIQYFELIA EFTSKALDIV KNKGYSIKNM
 LEIRKLESGA (SEQ ID NO:22). The Kvp40UvsY sequence is as follows: MKLQDLKAEY
 10 HEDVKIDTTA LETAAIRIPV LHAKWLAYRA DARQLLIKAE MKMEAVRKDR
 WLFYSGKHDD EVCDFIVEKS EMKYALAGDE ALQLAIARFQ HMKDVLSFIE
 EALKGISQMG FTIKHIIDNR KIESGIV (SEQ ID NO:23). The PSSM2UvsY sequence is
 as follows: MNLDKIQEMW ERDAVIDPDN LHDESLKIPQ LHSKYTYTVYN
 TVTLMREKAR EQYNKTRLER HNYTGTGKAPA EVYIEEPFGY KVREKDAIQR
 15 YMEADEKMSK IDLKIRYYDT TLKFLEEIIK NISNRTFQIK NAIEWNKFQA GM (SEQ
 ID NO:24). The PSSM4UvsY sequence is as follows: MNLEQIQEMW KKDSVIDNDL
 YCEESTKIPQ LHMRYMELYT TFGLMKKERE IEMKRLIREK WLYYK GKAPS
 SVYKELPFDL KLTTKEEVNM FIEGDDDV RK LQYKIEYVEQ CLNYLDGVLR
 QINNRFQIK NAIDWTKFQN GL (SEQ ID NO:25).

20 Figure 7 shows the primary sequence alignment of diverse gp32 proteins. The
 T4gp32 sequence is as follows: MFKRKSTAEL AAQMAKLNGN KGFSSSEDKGE
 WKLKLDNAGN GQAVIRFLPS KNDEQAPFAI LVNHGFKKNG KWIYETCSST
 HG DYDSCPVC QYISKNDLYN TDNKEYSLVK RKTSYWANIL VVKDPAA PEN
 EGKVFKYRFG KKIWDKINAM IAVDVEMGET PVDVTCPWEG ANFVLKVKQV
 25 SGFSNYDESK FLNQSAIPNI DDES FQKELF EQMV DLSEMT SKDKFKSFEE
 LNTKFGQVMG TAVMGGAAAT AAKKADK VAD DLDAFNVD DF NTKTEDDFMS
 SSSGSSSSAD DTDLDLLND L (SEQ ID NO:26). The T6gp32 sequence is as follows:
 MFKRKSTAEL AAQMAKLNGN KGGFSSSEDKG EWKLKLDNAG NGQAVIRFLP
 SKNDEQAPFA ILVNHGFKKNG KWIYETCSS THGDYDSCPVC QYISKNDLY
 30 NTDNKEYSLV KRKTSYWANI LVVKDPAAPE NEGKVFKYRF GKKIWDKINA
 MIAVDVEMGE TPVDVTCPWE GANFVLKVKQ VSGFSNYDES KFLNQSAIPN
 IDDES FQKEL FEQMV DLSEM TSKDKFKSFE ELSTKFSQVM GTAAMGGAAA

- TAAKKADKVA DDLDAFNVDD FNTKTEDDFM SSSSGSSSSA DDTDLDDLLN DL
 (SEQ ID NO:27). The Rb69gp32 sequence is as follows: MFKRKSTADL AAQMAKLNGN
 KGFSSSEDKGE WKLKLDASGN GQAVIRFLPA KTDDALPFAI LVNHGFKKNG
 KWIETCSST HGDYDSCPVC QYISKNDLYN TNKTEYSQK RKTSYWANIL
 5 VVKDPQAPDN EGKVFYRFG KKIWDKINAM IAVDTEMGET PVDVTCPWEG
 ANFVLKVKQV SGFSNYDESK FLNQSAIPNI DDESFKELF EQMVDLSEMT
 SKDKFKSFEE LNTKFNQVLG TAALGGAAAA AASVADKVAS DLDDFDKDME
 AFSSAKTEDD FMSSSSSDDG DLDDLLAGL (SEQ ID NO:28). The Aeh1gp32 sequence
 is as follows: MSIFKRKDPS QLQQQLAAFS AKKGFESDAT EWKLTQGKDG
 10 NGAAVIRFLP AKGDNATTFV KLVNHGFQRN GKWYIENCSS THGDYDNCPA
 CQWIKQNWD YNVEADKKAM YASGVTRKTA FWANILVIKD PANPDNEGKV
 FKFRFGKKIM DKIQAEVDVN TDLGEEPCDV TCPFEGKNFT IKIKKVGGNN
 NYDDSVFGKQ SQIANIEDEA YQAQLFEQMH DIMDLIAKDK FKSMECLTTV
 FNRVMGAEKR SNARAADDFE KQMEQFENTP ASKPEKEDDD VPFNTPSAGT
 15 VDTDLDDLLN EI (SEQ ID NO:29). The Rb43gp32 sequence is as follows:
 MSFFKRQDPT KLQEQVAALK GSSGFQKDEK EWKLTLDQK NGSVIRFLP
 NRSDDLAFLV RIVNHSFKKQ NQWYIENCPS THGDYDGCPV CQYITDNDLF
 EKAKANKGGE ADKLLGQIGR KQSFWANILV IKDPGNPENE GKVFKFRFGK
 KIMDKITATI AGNPDLDEPG IAVTCPFAGA NFTLKAKKVG DWPNYDDSTF
 20 GVPGPIKGID DEAVQKAIFE GMSDLRPITA PDQFKPTAEL TAKFTKVFGG
 GAAMGAGSSA GADLDSELNS FDADLKNFDN GNQSGSVKES GGVNQLNVGG
 SVPEDDTPFD LDNTSGDDDL DKLLDL (SEQ ID NO:30). The Kvp40gp32 sequence is
 as follows: MFKRKSPAQL QEKLEKMSSK KSFDNADEWK LTDTKLGNCS
 AVIRFLPAKG EDDLPFVKIF THGFKENGW FIENCPSTID LPCPCCAANG
 25 ELWKTEIEDN QNIARKRKRT LSYWANIVVI KDDAAPENEG KVFKYRFGKK
 ILDKITQAAQ ADEDLGVPGM DVTCVFDGAN FSLKAKKVSG FPNYDDSKFG
 PSTELYGGE AKLKEVWDAM HDLNAIAPS AFKSEAELQK RFLQVTGAAQ
 PKASAAQNLE AQLNTSAPAQ ANAPKAAAKP AAASVDVDSE PVTDSVDEL
 DALLADLELG DD (SEQ ID NO:31). The PSSM2gp32 sequence is as follows:
 30 MSFAKLKKQS KLGSLTQKLV KEVEKMNTG GQGDDRLWKL EVDKGGNGYD
 VIRFLPAPDG EDLPFVKLYS HAFQGPWWY IENSLTTLGQ KDPVSEFNSQ
 LWNNGTDAGK DTARKQKRKL TYISNIYVVK DPANPENEGK TFLYKYGKKI

FDKLTAA MQP EFEDDEAIDP FDFWQGANFK LKAKNVAGYR NYDSSEFAAT
 SALLDDDDAM EAIWKKEYSL AELVATDQFK SYDELKTRLG YVLGNKPVRN
 DAETVEQEVE DVRASAPVVE TVESVSRSSA TEDEDDTLSY FAKLAES (SEQ ID
 NO:32). The PSSM4gp32 sequence is as follows: MSFASLK KAA SAGSTLSKLT
 5 QEIEKINQPQ QNNSADERFW KPELDKSGNG FAVIRFLPAP EGEEMPWAKV
 WSHAFKGP GG QWYIENSLTT IGKDDPVGEY NRELWNSGKE SDKNIARAQK
 RKLSYYSNIY VVSDPAHPEN EGKVFLYKYG KKIFDKLVEA MQPAFADETP
 LDPFNFWKGA DFKLKIRKLD GYWNYDKSEF AATSTLGGFD DSKLESIWKE
 GYSLTEFESA KNFKDYDALK KRLDLVLGLT IPHPTTEDES LEDLSEGKTP
 10 SSWGQEV SDF REKAVASSPV QDEEDTLSYF SRLAEED (SEQ ID NO:33).

Figure 8 is a picture of an ethidium bromide stained agarose gel showing RPA products using T6 UvsX and T4 UvsX for amplification. Rs8179145-2 is (SEQ ID NO:34) and RS8179145-3 is (SEQ ID NO:35).

Figure 9 is a graph showing a comparison the kinetic behaviour of T6 and T4 UvsX in
 15 an RPA reaction using SYBR green dye.

Figure 10 is graph showing a comparison of kinetic behaviour of T6 and T4 UvsX in an RPA reaction using a fluorescent probe.

Figure 11 is a schematic layout of novel, engineered T6 UvsX protein constructs of the present invention.

20 Figure 12 a graph showing a comparison of the kinetic behaviour of T6 UvsX H66S and wild type T6 UvsX using a fluorescent probe.

Figure 13 is a graph showing a comparison of the kinetic behaviour various T6 UvsX mutants in an RPA reaction using a fluorescent probe.

Figure 14 is graph showing a comparison of the DNA amplification by Rb69
 25 components in an RPA reaction. Samples were analyzed using SYBR green dye.

Figure 15 is a graph showing a comparison of the DNA amplification by Aeh1 components in an RPA reaction. Samples were analyzed using a fluorescent probe.

Figure 16 is a graph showing a comparison of the DNA amplification by Aeh1 components and the effect of salt titration in an RPA reaction. Samples were analyzed using
 30 SYBR green dye.

Figure 17 is graph showing a comparison of the kinetic behaviour of the Aeh1 system to the T4 system in an RPA reaction,. Samples were analyzed using a fluorescent probe.

Figure 18 is a graph showing Aeh1 UvsX and UvsY and heterologous gp32 can amplify DNA using an RPA reaction. Samples were analyzed using SYBR green dye.

Figure 19 is a picture of an ethidium bromide stained agarose gel showing DNA amplification in an RPA reaction using heterologous reaction components: Rb69, gp32 and Aeh1 UvsX, and Aeh1 UvsY.

Figure 20 is a schematic representation of novel Rb69 engineered constructs.

Figure 21 is a schematic representation of additional novel Rb69 engineered constructs. The sequences, from top to bottom are SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, and SEQ ID NO:42.

Figure 22 is a graph showing a comparison of the kinetic behaviour of Rb69 and Rb69 H64S in an RPA reaction. Samples were analyzed using SYBR green dye.

Figure 23 is a graph showing a comparison of the effects of Rb69 gp32 titration on RPA using wildtype Rb69 UvsX or mutant Rb69 UvsX H64S. Samples were analyzed using SYBR green dye.

Figure 24 is a graph showing a comparison of the kinetic behaviour of mutant Rb69 H64S UvsX protein to wildtype Rb69 UvsX in an RPA reaction. Samples were analyzed using a fluorescent probe.

Figure 25 is a graph showing mutant Rb69 H64S UvsX is functional in RPA over a range of Rb69 gp32 concentrations (300, 400, or 500 ng/μl of Rb69 gp32 protein). Samples were analyzed using a fluorescent probe.

Figure 26 is a graph showing a titration of mutant Rb69 H64S UvsX in RPA (00, 150 or 200 ng/μl Rb69 H64S UvsX). Samples were analyzed using a fluorescent probe.

Figure 27 is a graph showing additional titration of mutant Rb69 UvsX in RPA (60, 80 or 100 ng/μl Rb69 H64S UvsX). Samples were analyzed using a fluorescent probe.

Figure 28 is graph showing the effectiveness of Rb69 gp32 in RPA reactions with T4 UvsX and UvsY. Samples were analyzed using a fluorescent probe.

Figure 29 is a graph showing a comparison of the kinetic behaviour of T4 and the Rb69 UvsX/UvsY system in RPA when Rb69 gp32 is used at high concentrations. Samples were analyzed using a fluorescent probe.

Figure 30 is a graph showing the kinetic behaviour of mutant Rb69 UvsX H64T in RPA. Samples were analyzed using a fluorescent probe.

Figure 31 is a graph showing ATP titration when using Rb69 UvsX in RPA. Samples were analyzed using a fluorescent probe.

Figure 32 is a graph showing the effect of T4 gp32 on Rb69 UvsX and UvsY in RPA. Samples were analyzed using a fluorescent probe.

5 Figure 33 is a graph showing a comparison of the kinetic behaviour of mutant Rb69 UvsX constructs having modifications to the C-terminus, in an RPA reaction. Samples were analyzed using a fluorescent probe.

Figure 34 is a graph showing a comparison of the kinetic behaviour of additional mutant Rb69 UvsX constructs having modifications to the C-terminus, in an RPA reaction.

10 Samples were analyzed using a fluorescent probe.

Figure 35 is a graph showing the titration of PEG 35,000 when using mutant Rb69 UvsX H64S 2xLDE in an RPA reaction. Samples were analyzed using a fluorescent probe.

Figure 36 is a schematic of novel, engineered, hybrid UvsY constructs.

Figure 37 is a graph showing the kinetic behaviour of novel UvsY hybrid constructs with T4 UvsX and T4 gp32 in RPA. Samples were analyzed using a fluorescent probe.

Figure 38 is a graph showing a comparison of novel UvsY hybrid constructs with Rb69 UvsX and Rb69 UvsY in RPA.

Figure 39 is a graph showing the kinetic behaviour of mutant Rb69 UvsX H64S/T6-1 2xLDE in RPA. Samples were analyzed using a fluorescent probe.

20 Figure 40 is a graph showing the titration of Rb69 gp32 in the presence of mutant Rb69 UvsX H64S/2xLDE in RPA. Samples were analyzed using a fluorescent probe.

Figure 41 is a graph showing the kinetic behaviour of mutant Rb69 UvsX H64S/2xLDE and Rb69 H64S/F69M/G70S/T6-1/2xLDE in RPA. Samples were analyzed using a fluorescent probe.

25 Figure 42 is a graph showing the kinetic behaviour of mutant Rb69 H64S T68S/L68N/T4/2xLDE in RPA. Samples were analyzed using a fluorescent probe.

Figure 43 is a graph showing the effect of titration of Rb69 gp32 when using mutant Rb69 UvsX H64S T67S/L68N/T4/2xLDE in RPA. Samples were analyzed using a fluorescent probe.

30 Figure 44 is a graph showing the activity of mutant Rb69 UvsX H64S/T67S/L68N T4 2xLDE protein with T4 gp32 in RPA. Samples were analyzed using a fluorescent probe.

Figure 45 is a graph showing the activity of Rb69 UvsX chimeras containing DNA-binding loops from phage 133, cyanophage, and Aeh1 in RPA. Samples were analyzed using a fluorescent probe.

Figure 46 is a graph showing the activity of mutant Rb69 UvsX H64S T6 2xLDE in RPA. Samples were analyzed using a fluorescent probe.

Figure 47 is a picture of an ethidium bromide stained gel showing amplified DNA products from RPA reactions using 0.1mM, 0.5mM, 1mM, 2mM, 3mM manganese.

Figure 48 is a graph showing DNA amplification using S.Aureus Pol I in RPA. Samples were analyzed using SYBR green dye.

Figure 49 is graph showing heparin the onset of noise detection using water as a control in RPA reactions. Samples were analyzed using SYBR green dye.

Figure 50 is a graph showing improved resolution of low copy target numbers by the use of Heparin in RPA reactions. Samples were analyzed using a fluorescent probe.

Figure 51 is graph showing DNA amplification using blocked primers in RPA. Samples were analyzed using a fluorescent probe.

Figure 52 is a picture of an ethidium bromide stained agarose gel showing RPA products using T6 H66S UvsX and Rb69 gp32 in the presence or absence of UvsY loading agent.

Figure 53 is another picture of an ethidium bromide stained agarose gel showing RPA products using T6 H66S UvsX and Rb69 gp32 in the presence or absence of UvsY loading agent.

Figure 54 is a picture of an ethidium bromide stained agarose gel showing DNA amplification of small genomic DNA targets using T6 H66S UvsX and Rb69 gp32 in the presence or absence of UvsY loading agent.

Figure 55 is a picture of an ethidium bromide stained agarose gel showing DNA amplification of complex genomic DNA targets using T6 H66S UvsX and Rb69 gp32 in the presence or absence of UvsY loading agent.

Figure 56, is a picture of an ethidium bromide stained agarose gel showing RPA products using T6 H66S UvsX and Rb69 gp32 in the presence or absence of UvsY loading agent and in the presence or absence of PEG.

Figure 57 is a picture of an ethidium bromide stained agarose gel showing RPA products using T6 H66S UvsX with T4 gp32 or Rb69 gp32 in the presence or absence of UvsY loading agent.

Figure 58 is a picture of an ethidium bromide stained agarose gel showing RPA products using T6 H66S UvsX with Rb69 gp32 or Aeh1 gp32 in the presence or absence of UvsY loading agent.

Figure 59 is a picture of an ethidium bromide stained agarose gel showing RPA products using T6 H66S UvsX or T4 UvsX with Rb69 gp32 in the presence or absence of UvsY loading agent.

Figure 60 is a picture of an ethidium bromide stained agarose gel showing RPA products using T6 H66S UvsX or T4 UvsX with T4 gp32 in the presence or absence of UvsY loading agent.

Figure 61 is a graph showing DNA amplification using T4 UvsX or T6 H66S UvsX with Rb69 gp32; in the presence or absence of UvsY loading agent. Samples were analyzed using a fluorescent probe system.

Figure 62 is a picture of an ethidium bromide stained agarose gel showing RPA products using T6 UvsX or T6 H66S UvsX with Rb69 gp32 in the presence or absence of UvsY loading agent.

Figure 63 is a picture of an ethidium bromide stained agarose gel showing RPA products using T6 H66S UvsX or Rb69 UvsX with Rb69 gp32 in the presence or absence of UvsY loading agent.

Figure 64 is a picture of an ethidium bromide stained agarose gel showing RPA products using Rb69 UvsX or Aeh1 UvsX with Rb69 gp32 in the presence or absence of UvsY loading agent.

Figure 65 is a picture of an ethidium bromide stained agarose gel showing RPA products using T6 H66S UvsX or Rb69T6loop2H64S UvsX with Rb69 gp32 in the presence or absence of UvsY loading agent.

Figure 66 is graph showing the results of the effects of titrating Rb69 gp32 in an assay designed to detect gp32 activity. Samples were analyzed using a fluorescent probe.

Figure 67A-67C are graphs comparing the activity of T4, Aeh1 and Rb69 gp32 molecules in an assay designed to detect gp32 activity. Samples were analyzed using a fluorescent probe.

Figures 68A-68C are graphs comparing the upper temperature limits of T4, Aeh1 and Rb69 gp32 molecules in an assay designed to detect gp32 activity. Samples were analyzed using a fluorescent probe.

Figure 69 is a graph showing the comparison of DNA amplification in RPA reactions using T4 UvsX with Rb69 gp32, in the presence and absence of UvsY loading agent. Samples were analyzed using a fluorescent probe.

Figure 70 is an additional graph showing the comparison of DNA amplification in RPA reactions using T4 UvsX with Rb69 gp32, in the presence and absence of UvsY loading agent. Samples were analyzed using a fluorescent probe.

Figure 71 is a picture of an ethidium bromide stained agarose gel showing RPA products using T4 UvsX and Rb69 gp32 in the presence or absence of UvsY loading agent.

DETAILED DESCRIPTION OF THE INVENTION

This invention constitutes novel enabling data on the use of diverse, hybrid and engineered recombinase enzymes. The utility of a variety of recA/UvsX-like recombination proteins and associated recombination factors for carrying out RPA reactions is shown. Surprisingly, it was discovered that variant recombinases (e.g., novel engineered chimeric and mutant recombinases) and their associated components display differences in kinetics, differences in optimal PEG concentrations and SSB concentrations, and differences in dependence on recombinase loading factors. Furthermore, the novel chimeric and mutant proteins of the invention have permitted the elucidation of specific peptide regions that profoundly influence these behaviours.

The origin of some of the observed variation, and location of some key amino acids residues influencing activities in RPA assays is described herein. Particularly important are a mobile DNA-binding loop, as well residues in the Walker A motif found in ATPases.

Notably it was discovered that the peptide corresponding to DNA binding loop 2 in *E.coli* RecA is very important, and that this peptide is generally unrelated to *E.coli* RecA, and quite variant among RecA/UvsX-like proteins from the myoviridae. Surprisingly, it was discovered that the T6 UvsX protein, and derivatives of it, display very significant UvsY-independent activity in RPA reactions. This UvsY-independent activity may also be extended to other UvsX species under conditions which particularly favour UvsX-loading but is most obvious for T6 and its derivatives. This analysis has permitted the engineering of altered T6 and Rb69 UvsX recombinase proteins for use in RPA, and has set the stage for further optimization and

the development of engineered super-recombinases for the RPA system. Surprisingly, T6-derived recombinases show only partial requirement for loading proteins, albeit loading proteins improve reaction performance and robustness. Hybrid proteins can be utilized which display altered activities in the RPA process. Systems comprising heterologous combinations of recombination components may also be effectively used.

Additional components and conditions to improve RPA reactions are also provided herein. For example, the present invention provides other crowding agents which impart similar or even greater effects than Carbowax 20M (PEG compound) on RPA reactions. The inclusion of crowding agents, in particular those having a molecular weight of at least 10,000 and less than 100,000 was found to be highly stimulatory in RPA reactions. Such crowding agents include but are not limited to polyethylene glycol, polyethylene oxide, polyvinyl alcohol, polystyrene, Ficoll, dextran, PVP, and albumin. In particular, PEG molecular weight 35,000 was found to be very effective in RPA reactions. The present invention also provides the use of heparin in RPA reactions, as an agent to reduce the level of non-specific primer noise, and the ability of *E.coli* exonuclease III or *E.Coli* exonuclease IV to rapidly polish 3' blocking groups or terminal residues from recombination intermediates. Additionally, manganese ions are shown to be able to replace Magnesium, but at much lower concentrations.

Further, the present invention provides the use alternative polymerases capable of strand displacement synthesis for use in RPA reactions, including repair class polymerases, and polymerases which lack proof-reading activity. Surprisingly, the large fragment, not the full protein, of bacterial polymerase I repair enzymes which bear homology to the Pol I class of *E.coli*, *Bacillus subtilis*, and *Staphylococcus aureus* were found to be effective in RPA reactions, thus extending the repertoire of polymerases shown to be effective and further supporting the view that repair class, strand-displacing, polymerases from prokaryotes (and possibly phage) are generally effective.

Brief description of RPA

RPA is a method (process) for amplifying DNA fragments. RPA employs enzymes, known as recombinases, that are capable of pairing oligonucleotide primers with homologous sequence in duplex DNA. In this way, DNA synthesis is directed to defined points in a sample DNA. Using two gene-specific primers, an exponential amplification reaction is initiated if the target sequence is present. The reaction progresses rapidly and results in

specific amplification from just a few target copies to detectable levels within as little as 20-40 minutes.

RPA reactions contain a blend of proteins and other factors that are required to support both the activity of the recombination element of the system, as well as those which support DNA synthesis from the 3' ends of oligonucleotides paired to complementary substrates. The key protein component of the recombination system is the recombinase itself, which may originate from prokaryotic, viral or eukaryotic origin. Additionally, however, there is a requirement for single-stranded DNA binding proteins to stabilize nucleic acids during the various exchange transactions that are ongoing in the reaction. A polymerase with strand-displacing character is required specifically as many substrates are still partially duplex in character. Reduction to practice has established that in order to make the reaction capable of amplifying from trace levels of nucleic acids precise in vitro conditions are required that include the use of crowding agents and loading proteins. An effective system comprising bacteriophage T4 UvsX recombinase, bacteriophage T4 UvsY loading agent, bacteriophage T4 gp32 and *Bacillus subtilis* polymerase I large fragment has been reported earlier.

Analysis of Key Residues and Engineering Novel Recombinase Proteins

In an effort to learn more about the optimal conditions and proteins for performing RPA reactions efforts to clone and produce RecA/UvsX-like proteins from the myoviridae bacteriophages which are relatives of the T4 bacteriophage were made. Additionally other key protein components were identified, which might be required for RPA reactions from each respective phage, e.g., equivalents to the gp32 protein and the UvsY protein. Figure 1 shows a schematic representation of clones used to generate variant UvsX, UvsY and gp32 proteins. Hexahistidine tags were engineered at the N or C termini via incorporation of extra bases in oligonucleotides into PCR amplification primers used in their cloning. Templates were genomic phage DNA. T6 was obtained from the DSMZ stock centre in Germany, while Rb69, Aeh1 and KVP40 phages were obtained from the Institute Felix D'herelle in Canada.

A comparison of the biological activity of these proteins and analysis of the relationship of any biochemical differences to variation in the amino acids sequence of these proteins was made. Although none of the bacteriophage UvsX or UvsY proteins have been crystallized (or are unavailable in public databases, UvsX proteins are close relatives of bacterial RecA proteins for whom the structure is known. It has been hypothesized that RecA and UvsX originated from a common ancestor (Story et al., 1993). Although RecA and UvsX

proteins share only weak homology at the primary sequence level, they show very similar geometries and pitches when assembled onto DNA and share blocks of homology comprising the potential subunit interfaces. They also share other features associated with bacterial RecA proteins such as acidic C-terminal residues likely involved in modulating DNA affinity on duplex and single-stranded DNA (Benedict and Kowalczykowski, 1988). As described herein, UvsX proteins were modeled onto the known RecA protein sequence using a standard primary protein sequence alignment as template. This allowed the effects of primary peptide sequence variation to structural position and known biological function of regions involved in DNA binding, ATP binding and hydrolysis, subunit interface, etc., to be observed.

10 *RecA and T4 UvsX*

Figures 2 and 3 show a primary sequence alignment of bacteriophage T4 UvsX with *E.coli* RecA, and a representative 3-D structure of a model of an active *E.coli* RecA filament. These two proteins share 23% identity and are 43% similar at the primary sequence level. Various key regions of the RecA molecule which are implicated in biological activity and relevant to the discussion here are indicated on the alignment and the structure. The regions involved in binding and hydrolysing nucleotides are found intimately associated with the face of the protein involved in contacting the DNA backbone. Note that key residues defining the so-called Walker A motif (found in all ATP-hydrolysing enzymes) are found in both proteins. The Walker A consensus is often stated as A/G XXXXGK S/T (SEQ ID NO:43), where X is any amino acid (Walker et al., 1982). The *E.coli* RecA protein Walker A motif perfectly matches this consensus, while T4 UvsX notably lacks the second glycine immediately preceding the lysine. Most phage UvsX proteins other than T4 also lack this second glycine (see Figure 5), having a phenylalanine instead, however this is not the case for the somewhat more divergent recombinases of cyanophages SSM2 and SSM4. These latter proteins do possess the second glycine, and on the whole significantly more closely resemble RecA with regard to the Walker A sequence.

Other peptide sequences of interest for later discussions include those regions described as DNA binding loops 1 and 2 in *E.coli* RecA. These loops have been described as highly mobile, are implicated in direct contacts to DNA (Malkov and Camerini-Otero, 1995), and also as participating in the nucleotide hydrolysis process (Voloshin et al., 2000). It is thus significant to note that both the DNA binding loops (disordered in some crystal structures), and the Walker A motif are all located in close proximity to one another on a common face of

the protein. One can readily imagine that the dependence of ATP interaction for DNA binding, and the concomitant stimulation of ATP hydrolysis caused by DNA binding, are intimately interdependent processes involving direct interactions between these various peptides, ATP and DNA.

5 A last region of interest is the very C terminus of the *E.coli* RecA and T4 UvsX proteins. In both cases there is an acidic peptide sequence. This has previously been shown to influence the DNA binding properties of *E.coli* RecA, in particular when removed promoting stronger binding to double-stranded DNA and a reduction in dependence in magnesium ions and various salt and pH conditions (Eggler et al. 2003; Lusetti et al. 2003). Notably removal
10 of this acidic sequence may decrease the frequency with which disassembly of recombinase filaments occurs. In earlier work, it was reported that removal of this acidic sequence from either RecA or T4 UvsX altered the activity of the proteins in RPA reactions having a generally detrimental effect which may result from undesirably high DNA affinity on duplex substrates (Piepenburg et al. U.S.S.N. 10/931,916).

15 *T4 vs T6 UvsX protein*

An unexpected number of amino acid substitutions

A number of UvsX-like protein molecules are aligned in Figure 5. The T6 UvsX protein was cloned, sequenced, and expressed in *E.coli* with a histidine tag sequence at the C terminus. A similar draft sequence of the T6 UvsX protein was discovered in a database
20 provided at Tulane University. A surprising discovery was that quite a number of amino acid residues were variant between T4 and T6 UvsX proteins. There were 38 substitutions between the two proteins and a 2 amino acid insertion at the N-terminus. The reason that this significant level of heterology was a surprise is that T2, T4, and T6 (the so-called T-even phages) are regarded as fairly close relatives of one another. Oddly, all the substituted amino
25 acid residues were confined to more-or-less the N-terminal half of the protein, while the C-terminal half was completely conserved. This seemed particularly odd because when UvsX relatives from more diverged myoviridae members were studied it was noted that other regions such as the last C-terminal 30-40 residues were the least conserved. It was also noted that the primary DNA sequence was fairly well-conserved in the coding sequence for the C-
30 terminal half of the protein with few base changes even on wobble positions, while the N-terminal half showed concentrated clusters of base changes. Indeed many of the substituted amino acids required 2 base changes to achieve the amino acid substitutions observed. As

described below, some of these substitutions have occurred in regions important for function of the recombinase, and it is proposed that rather than supporting a model of mutations occurring principally at silent positions, in this case many substitutions may have been selected due to conferring measurable biochemical variation to the polypeptide.

5 *Relative activity of T4 and T6 UvsX proteins*

The activity of the T6 UvsX protein in DNA amplification assays was tested and monitored in real-time with a fluorescent probe or minor-groove binding dye, as well as some experiments in which products at end-point were monitored on agarose gels. In these experiments gp32 and UvsY proteins from T4 were employed. This approach was taken as
10 the gp32 and UvsY proteins from T4 and T6 appeared very similar. T6 UvsY was sequenced and only 2 highly conservative substitutions were found (see Figure 4). T6 gp32 had only 4 substitutions, and a single amino acid insertion. It was determined that the T6 UvsX protein was indeed active and worked effectively to amplify targets in this heterologous system. When assayed on agarose gels there was no significant consistent differences between
15 reactions performed with T4 and T6 UvsX proteins (90 minute reactions) (small differences in final product accumulation were observed here, but were not consistent and may arise through pipetting inaccuracies) (see Figure 8). However, when assayed in real-time using SYBR-green or with a probe-based approach, a measurable difference in reaction kinetics was observed. Reactions performed with T6 UvsX were consistently shallower in the curve
20 of signal accumulation than those performed with T4 UvsX, although generally the time at which the signal threshold was crossed were similar (see Figures 9 and 10 showing comparison of T4 and T6 UvsX amplification kinetics using SYBR-green or a probe). Without intending to be bound by any theory, the reproducibility of this effect appears to have been underpinned by real biochemical differences between these 2 proteins. Note here
25 however that one concern should be raised about the interpretation of experiments performed with a probe-based system. In order to generate strong signals in probe-based experiments, an asymmetric ratio of amplification primers were employed to encourage an excess of single-stranded DNA complementary to the probe late in the reaction. Should variant recombinases influence the ability of this single-stranded DNA to interact with the probe then it may mask
30 signals generated in this system and lead to lower overall fluorescence. This effect could have a mechanistically different origin to a similar response caused by poorer overall

amplification. In either case, however, it would reflect biochemical differences in the amplification components.

Source of variability between T4 and T6 UvsX

Walker A motif

5 In an effort to understand a possible relationship between the differing primary amino acid sequences of T4 and T6 UvsX and the observed biochemical differences the known structural and functional information available for RecA was studied, and the information was translated to the phage proteins. Of particular interest were the regions potentially involved in DNA binding and nucleotide hydrolysis. As discussed earlier, the affinity of
10 recombinases for ssDNA and dsDNA, and their turnover rates which are related to ATP hydrolysis rate, are factors likely to critically affect the behaviour of RPA reactions. Thus, the sequence of, and surrounding, the so-called Walker A motif (or 'P-loop') (consensus normally described as A/G XXXXGK S/T (SEQ ID NO:43)) which is highly conserved among most known (non-cyanophage) UvsX-like proteins, but is slightly eccentric in that it
15 lacks the second glycine of the canonical Walker A motif (see Figure 5, sequence GPSKHFKS (SEQ ID NO:44) in most proteins and APSKHFKT (SEQ ID NO:45) in Rb69), and is slightly different in T4 UvsX (GPSKSHFKS(SEQ ID NO:46))) was of particular interest. This motif is implicated in the binding and hydrolysis of ATP, possessing residues involved in co-ordinating the triphosphate backbone, and polar residues implicated in
20 stimulating hydrolysis. T4 UvsX possesses a serine residue at position 64 which is a histidine in all other UvsX proteins except the distant cyanophage homologs (which have a P-loop more reminiscent of RecA proteins). It was noted that this novel arrangement resulted in the generation of a new lysine-serine dipeptide in the middle of the Walker A motif, a feature normally found only at the C-terminus of the motif, and hence a re-iteration. Crucially the
25 lysine and serine (or threonine) residues of the Walker A motif are key to the co-ordination of the gamma phosphate (lysine) and hydrolysis of the phosphate-phosphate bond (serine/threonine). It was known from early studies that T4 UvsX demonstrated the unusual property that the protein hydrolysed ATP to AMP and pyrophosphate, as well as to ADP and phosphate, the more traditional reaction (Formosa and Alberts, 1986). This raised the
30 question whether this catalytic plasticity was imparted by this central lysine-serine dipeptide which could perhaps co-ordinate the beta-phosphate and catalyse hydrolysis of the alpha-beta phosphate-phosphate bond in a manner equivalent to the more traditional reaction (analysis of

the RecA protein structure suggested that these central residues might be appropriately positioned, see Figure 3). If true then it was anticipated that non-T4 UvsX proteins would not generate AMP and pyrophosphate, and this could have significant implications on their relative behaviours in RPA reactions. For example, in T4 UvsX, this activity might increase the overall total ATP hydrolysis activity with implications for the degree of dynamicity of the recombinase in the reaction. Also, as ATP and ADP are reported to be associated with different nucleoprotein helical pitches (Ellouze et al., 1995), so AMP might promote yet a third pitch which could be significant. Thus, this variant residue might underpin some or all of the variation observed between T4 and T6 UvsX.

Mutant T6 proteins were generated in which the histidine residue was replaced with serine at the equivalent central Walker A motif position as found in T4. This resulted in a protein which appeared improved relative to the original T6 protein sequence. In a variety of experiments involving sensing product accumulation in real-time, the slope was steeper and maximal signal generated was higher for the mutant T6 protein (Figure 12). It was concluded that this mutation directly benefits the behaviour of RPA reactions, particularly late in the reaction. This may result from one or more of several sources; (i) The recombinase may less efficiently bind duplex DNA, thus suffering less from out-titration of recombinase by product, (ii) The recombinase may hydrolyse ATP more effectively on duplex DNA, thus recycling more efficiently from duplex DNA, (iii) the hydrolysis the generate AMP and PPi from ATP may be associated with a new nucleoprotein pitch which is useful for maintaining high dynamic activity late in the reaction. Other explanations are, of course, possible.

Residues C terminal to Walker A motif

Despite significant improvement in the activity of T6 UvsX once histidine 66 had been mutated to serine, the protein still seemed to remain slightly different in behaviour to the T4 UvsX. Thus, other amino acids were examined. As mentioned earlier, the 38 amino acid substitutions between T6 and T4 are clustered in the N-terminal half of the protein.

Substitutions were found in several places that might be influential, namely residues directly C-terminal to the Walker A motif as well as those in the putative mobile DNA-binding loops (see more below). Figure 5 shows that T6 has 2 amino acids directly after the Walker A motif, namely methionine 71 and serine 72, that are different to T4 in which these residues are phenylalanine and glycine. In Figure 3, Panel B, the putative position of the T4 residues phenylalanine (F69) and glycine (G70) are indicated (assuming similar positioning in T4

UvsX as in *E. coli* RecA). Note that they are very close to the other important residues of the Walker A motif (or 'P' loop), and also to the putative mobile DNA binding loop2 whose beginning and end is indicated.

These variant residues were mutated in T6 UvsX to generate clone T6UvsX M71F/S72G the protein was made. This protein was tested in real-time assays and was found to be totally inactive (Figure 13). It was concluded that one or both of these residues are non-substitutable in isolation and that they must have a biochemical interaction with other substituted residues which are also altered in T6 UvsX to compensate and allow correct folding and/or activity. Further evidence that one or both of these residues confer measurable interaction with some other peptide regions is suggested by data presented later analysing Rb69 chimeras. In summary these two residues (M71 and S72), at least in isolation, are not silent substitutions between T4 and T6, nor do they in isolation appear responsible for conferring the slight difference between T4 and T6 UvsX.

DNA binding loop 1

Comparison of T4 and T6 peptide sequences suggested that those sequences likely comprising the equivalent of DNA binding loop 1 of *E. coli* RecA were generally very highly conserved between T4 and T6 UvsX (Figure 5). Nonetheless, 2 residues at the end of the putative region were variant, namely serine 164 of T6 which is valine in T4, and alanine 166 of T6 which is serine in T4. These residues were both mutated together in T6 to generate the clone T6 UvsX S164V/A166S. This protein was expressed, purified, and tested it in real-time assays. The first experiment performed with this protein is shown in Figure 13, in which it performs well, and slightly better than wild type T6. It was noted that in later experiments its behaviour seemed almost indistinguishable from wild type T6. Consequently, within the boundaries of error of the experiments, it is suggested that these substitutions are silent between T4 and T6 polypeptides and do not contribute significantly to the assayable characteristics addressed in these experiments.

DNA binding loop 2

One of the most interesting peptide sequences in *E. coli* RecA is the so-called mobile DNA binding loop 2. This peptide has been shown to possess DNA binding activity, even in complete isolation from the whole protein (Voloshin et al., 1996). The loop has also been variously implicated in stimulating ATP hydrolysis when recombinase is bound to DNA, and even to have a catalytic role in ATP hydrolysis (Voloshin et al., 2000). It was anticipated that

the equivalent sequence would be of great importance to UvsX function. Note however that this peptide is unrelated to the RecA peptide.

As illustrated in Figure 5, T6 and T4 have 3 substitutions in the region of the putative DNA binding loop 2 region. An additional alignment of all known UvsX-like proteins in this region is shown below. Sequences have been loosely grouped by similarity. An alignment of the RecA loop is shown in this region also.

DNA binding loop 2 sequences

10	T6	NHT	IETIEMFSKT	VMT	GG	(SEQ ID NO:47)
	RB3	NHT	IETIEMFSKT	VMT	GG	(SEQ ID NO:48)
	LZ2	NHT	IETIEMFSKT	VMT	GG	(SEQ ID NO:49)
	RB14	NHT	IETIEMFSKT	VMT	GG	(SEQ ID NO:50)
	RB32	NHT	IETIEMFSKT	VMT	GG	(SEQ ID NO:51)
15	133	NHT	LQTLEMFSKE	VMT	GG	(SEQ ID NO:52)
	T4	NHT	YETQEMFSKT	VMG	GG	(SEQ ID NO:53)
	AE65	NHT	YETQEMFSKT	VMS	GG	(SEQ ID NO:54)
	PHI 1	NHT	YETQEMFSKT	VMS	GG	(SEQ ID NO:55)
	RB49	NHT	YETQEMFSKT	VMS	GG	(SEQ ID NO:56)
20	RB16	CHT	YDTQEMYSKK	VIS	GG	(SEQ ID NO:57)
	RB43	CHT	YDTQEMYSKK	VIS	GG	(SEQ ID NO:58)
25	AEH1	AHT	YDTQEMYSKK	VVS	GG	(SEQ ID NO:59)
	KVP40	NHT	YQTQEIYSKT	VMS	GG	(SEQ ID NO:60)
	NT1	NHT	YQTQEMYSKT	VMS	GG	(SEQ ID NO:61)
30	PSSM2	NHT	YDVIGSYVPTK	EMG	GG	(SEQ ID NO:62)
	PSSM4	NHT	YDVVGAYIPTK	EMG	GG	(SEQ ID NO:63)
	SPM2	NHT	YDVVGSYVPTK	EMG	GG	(SEQ ID NO:64)
35	RB69	NHT	AMEIGGLYPKE	IMG	GG	(SEQ ID NO:65)
	JS98	NHT	AMEIGGMYPKE	IMG	GG	(SEQ ID NO:66)
	RECA	QI	RMKIGVMFGNPETTT	GG		(SEQ ID NO:67)
	Required RecA	**	* *	* *	**	
	Hydrolysis	o	o o	o		
40	Involvement					

It was noted that residue isoleucine 199 and isoleucine 202 were not only different in T6 (being respectively a tyrosine and glutamine in T4 UvsX), but were T4-like in many of the loops from other UvsX relatives. This latter observation suggested that they might not be trivially substituted. Furthermore it was noted that using the best possible alignment

generated with the RecA loop, isoleucine 199 corresponded to a RecA residue shown to be necessary for activity. The consequence of altering either I199 or I202 to the T4 equivalents was investigated. Mutant clones were generated and the proteins were expressed. Substitution of either I199 or I202 to their T4 equivalents completely inactivated the protein. This result was something of a surprise, but once again highlights the fact that these substitutions are not silent and have significant biological consequences. It was assumed that each of these substitutions in T6 UvsX is matched by at least one other compensating substitution elsewhere. Furthermore, all UvsX molecules with a loop length similar to T4 and T6 (see below) possess a tyrosine and glutamine like T4 at these positions apart from those in the T6 group and phage 133, and in these cases both residues are altered to either isoleucine (T6 group) or leucine (133). It was hypothesized that these particular residues have key interactions with one another and must be substituted in unison. To test this hypothesis, a double mutant T6 UvsX molecule was generated with both of these residues changed to the T4 equivalents. It was found that the double mutant protein also failed to demonstrate activity in amplification assays suggesting that other variant residues which are substituted between T4 and T6 underpin the substitution compatibility problem. This highlights the fact that a number of the substitutions between T4 and T6 UvsX proteins occur in non-silent positions and have real influence on protein biochemistry.

Amplification systems employing Rb69, Aeh1 and KVP40 proteins

Clones encoding the UvsX, UvsY and Gp32 proteins of bacteriophages Rb69, Aeh1 and KVP40 were generated, as indicated in Figure 1. Alignments of these 3 proteins are shown in Figures 5, 6, and 7 and include other known homologs. A possible error in the NCBI Genbank database was noted with regard to the Rb69 UvsY sequence. According to the database the Rb69 UvsY would have an N-terminal extension relative to the sequence shown here, however attempts to express this longer polypeptide were unsuccessful and lead to the re-examination of the sequence. It was noted that all other identifiable UvsY proteins begin at a near-identical point and that the database entry included a methionine at the equivalent position to the first methionine of the others. It was deduced that the automatic annotation software was erroneous. Probable errors in annotation were also identified for some of the cyanophage entries for UvsY and Gp32 which had been artificially truncated at the N terminus compared to the sequences shown in the alignments herein.

All of the proteins illustrated in Figure 1 expressed and purified robustly with the exception of KVP40 gp32. Only relatively limited amounts of this protein were recovered despite no apparent errors in the sequence of the clone. A possible source of this biochemical oddity was speculated. Study of the alignment of gp32 molecules shown in Figure 7 reveals that KVP40 is eccentric relative to T4, T6, Rb69, and Aeh1 gp32 molecules in the part of the primary sequence corresponding to residues implicated in co-ordinating Zinc atoms in T4 gp32. More specifically 4 residues have been implicated to be involved in binding zinc in T4 gp32, these are either histidine 64, cysteine 77, cysteine 87, and cysteine 90 (Qiu and Giedroc D.P., 1994) or Histidine 81 cysteine 77, cysteine 87, and cysteine 90 which were reported earlier (Giedroc et al., 1987). In T4, T6, Rb69, and Aeh1 gp32's these 4 residues are highly conserved with identical spacings and very high conservation of residues in general between histidine 64 and cysteine 90.

Zinc co-ordination has been shown to be critical for the cooperative behavior of T4 gp32 (Nadler et al., 1990), and the apoprotein does not support effective RPA reactions (see Piepenburg et al.). However KVP40 gp32 has significant disruption to the spacing of putative coordinating residues in the C-terminal half of this region, and little or no homology with other residues in T4, T6, RB69; and Aeh1 in this region. It was proposed that this disruption has altered the metal-binding properties of KVP40 gp32 relative to T4, T6, Rb69 etc. Without intending to be bound by any theory, it is possible that KVP40 no longer binds Zinc, or instead uses another metal atom such as Cobalt. It was noted that KVP40, a broad spectrum vibriophage, was isolated from a marine environment in which trace metal conditions may be different to those inhabited by coliphages. Without intending to be bound by any theory, perhaps an altered metal dependency and folding characteristics have influenced the efficiency of expression in *E.coli*. Furthermore it was noted that the cyanophage SSM2 and SSM4 putative protein sequences are conspicuous in the absence of any of the conserved coordinating cysteine residues. It was assumed that these gp32 molecules do not contain a zinc, or similar, metal atom. This is of some considerable interest as occasional problems in the activity of gp32 have been encountered, likely caused by co-purification of apoprotein, or by loss of zinc from the protein under poor storage conditions. Furthermore as gp32 loses the zinc atom when heat denatured, it has consequently has been of limited use in PCR or other techniques requiring a heat denaturation step. If the SSM2 and SSM4 gp32 proteins have engineered a way to have similar co-operative behavior without zinc atoms, and still have all

the other properties required for RPA, then they could be very useful agents for RPA or other techniques.

RPA with Rb69 proteins

RPA reactions were configured with Rb69 UvsX, Rb69 UvsY, and Rb69 gp32.

- 5 Limited investigation into optimal component concentrations established that reaction behavior was notably distinct from T4 or T6 UvsX-based systems. It was found that higher quantities of UvsY were required for optimal activity. Figure 14 shows amplifications performed with SYBR green and Figure 24 shows reactions monitored with a fluorescent probe system. Reactions work well but have slightly slower kinetics than T4 or T6 based
- 10 reactions. Oddities in the behavior of the Rb69 amplification system were noted. For example the amplification system was strangely very sensitive to overtitration of both Rb69 gp32 (see Figure 23), and sensitive to overtitration of Rb69 UvsX (see Figures 26 and 27). Both these sensitivities were distinctive and different from observations made with T4 (and T6) amplification systems. Significant efforts were made to address the underlying source of
- 15 these differences which are later described. However, it was noted that despite these variations, highly effective RPA reactions may be configured with Rb69 components, again confirming the generality of the RPA system and the possibility of using a wide range of recombinase agents and associated factors.

RPA with Aeh1 proteins

- 20 RPA reactions were configured with Aeh1 UvsX, Aeh1 UvsY, and Aeh1 gp32 (see Figures 15, 16, and 17). As with the Rb69 system it was found that the Aeh1 system was clearly functional, but showed differences to the T4 and T6 based systems. There appeared to be dependency on higher quantities of polyethylene glycol, and once again kinetics tended to be somewhat slower than observed with T4 and T6.

- 25 One observation that was made using both gel-based assays (Figure 19) and real-time assays (Figure 18) is that an amplification system could be configured that used Rb69 gp32 in combination with Aeh1 UvsX and Aeh UvsY, albeit perhaps not as robust as when all Aeh1 components are used. This interesting result suggests that the gp32 species used may not absolutely need to match the UvsX and UvsY species.

- 30 *RPA with KVP40 proteins*

KVP40 gp32 did not express robustly in *E.coli* under the conditions of growth and induction used. Consequently an amplification system using KVP40 components was unable

to be established. Nevertheless there is some reason to believe that KVP40 UvsX and UvsY may possess basic biochemical activities required for establishing RPA reactions. In one experiment KVP40 UvsX and UvsY were combined with either gp32 from Rb69, or gp32 from Aeh1. Under these conditions there was evidence of DNA synthesis and while a product of expected size was not generated the presence of apparently amplified primer artifacts lends support to the idea that recombination-mediated polymerase priming was occurring. This suggests partial functionality of this heterologous systems, and it is proposed that KVP40 might also in principle be adapted to a useful RPA system.

Rb69 chimeras

The source of some of the most marked differences in RPA reactions using Rb69 components rather than those of T4 and T6 are addressed herein. Figure 14 reveals one of the first oddities of the Rb69 system, namely that Rb69 seems to require more UvsY than the T4 or T6 systems. A second oddity is that the Rb69 system is very sensitive to the concentration of gp32 that is employed as revealed in Figure 23. Such a high degree of sensitivity was not noted for the T4 system. A third oddity is that the Rb69 RPA system is very sensitive to the concentration of UvsX employed as revealed in Figures 26 and 27, in particular suffering if excess protein is employed. Other peculiarities were discovered in addition to these as protein in heterologous mixtures were compared with other proteins. For example it was found that Rb69 UvsX could not tolerate T4 gp32 at all, while Rb69 gp32 worked very efficiently with T4 UvsX and T4 UvsY (Figures 28, 29, and 32). Similarly it was found that Rb69 UvsY would readily support amplification with heterologous T4 components (Figure 37), but when Rb69 UvsX was employed the type of UvsY used had a significant impact on the outcome of the experiment (Figure 38). Rb69 UvsY gave the highest stimulation, while T4 UvsY or hybrids between T4 and Rb6 UvsY were markedly less effective.

A possible explanation to rationalize the above data is presented herein. Without intending to be bound by any theory, it is suggested that Rb69 UvsX is mainly responsible for the variant behavior of the Rb69 amplification system. Perhaps Rb69 UvsX has relatively poor DNA binding behavior in comparison with T4 UvsX, at least under the salt, pH, and other conditions employed by us here. As a consequence perhaps Rb69 UvsX has relative difficulty in coping with the excess quantities of gp32 present in the system, being a poor DNA-binding competitor, and, as such it is more dependant on highly effective UvsY behavior, is inhibited by excessive gp32, and sensitive to the fecundity of the gp32 and UvsY

species employed which are presumably subtly different between Rb69 and T4 proteins (thus explaining why T4 UvsX is largely unaffected by the species of gp32 or UvsY used while Rb69 UvsX is affected).

This theory could account for most of the observations made about RPA reactions using RB69 components. However one aspect that is left unanswered by this is the question of why the reactions should be sensitive to overtitration of Rb69 UvsX, which on the face of it one would expect to help rather than hinder reaction kinetics. Without intending to be bound by any theory, perhaps a second factor that might be in play is that Rb69 UvsX may not support the hybridization of complementary oligonucleotides to one another. It is reported that RecA and UvsX support the hybridization of complementary oligonucleotides, a property essential to effective RPA reactions as strand displacement DNA synthesis must generate quantities of ssDNA that require conversion to duplex DNA via hybridization, not invasion, based priming. If true then the situation might be explained as follows: Rb69 UvsX has a low affinity for, or residence time on ssDNA, compared with T4/T6 UvsX which means that it competes poorly with excess gp32 (hence sensitivity to gp32 overtitration), however it also fails to support oligonucleotide hybridizations and thus encouraging overly high oligonucleotide-recombinase loading also leads to impaired amplification reactions as few primers are available for hybridization. Consequently a middle ground would have to be struck in which roughly half the primers are coated with UvsX and half are coated with gp32. That the maximal optimum RB69 UvsX concentration was found to be ~100 ng/ μ l, which is roughly half that required to saturate all primers in the reaction may be no coincidence.

Despite the above 'theory' there exist other reasonable explanations, and other data exists that is somewhat inconsistent with this model. For example gel analysis of Rb69 component-mediated amplifications (not shown here) reveal larger amounts of product DNA than is typically generated found with a T4-based system. Overall such reactions gave the impression of extremely high recombinase activity somewhat inconsistent with the interpretation that Rb69 UvsX has weak DNA-binding behavior. This suggests that Rb69 UvsX might show altered ssDNA/dsDNA partitioning relative to T4 or T6 UvsX, perhaps showing less inhibition by duplex DNA build-up.

Whatever the rationale for the differences in behavior of Rb69 and T4/T6 UvsX molecules, which are speculative at this time, one peptide region that is prime suspect in all of this is the putative mobile DNA binding loop2. Figure 5 showing the alignment of UvsX

proteins reveals how very unusual the Rb69 loop2 sequence is compared to its nearest homologous neighbors. Unlike T4, T6, Aeh1, KVP40, phage 133 (and all UvsX molecules apart from JS98 which is a close Rb69 relative), and the cyanophage proteins, the Rb69 loop 2 has a different number of amino acids and appears completely recoded in comparison to the others. This is most unexpected, and given the attention paid to this loop in studies of RecA, and the results described above regarding subtle alterations found in the T4 and T6 loops, it was anticipated that this variant loop sequence might underpin much of the measurable differences.

Other putative UvsX-like loop2 sequences and Walker A amino acids were employed and used to replace the Rb69 version. Additionally, changes to the acidic C-terminus of the protein were investigated. Figures 20 and 21 show schematic representations of clones that were generated in order to express mutant proteins. These experiments followed a temporal flow of investigation which means that most data was generated by successive steps of alteration of clones which were generated in an Rb69 protein backbone.

Initially the histidine in the Walker A motif was substituted for serine as was done for T6. Figures 22 and 24 show experiments performed to compare Rb69 UvsX wild type with Rb69 H64S. Figures 22 and 24 show that Rb69 H64S performs better than the wild type equivalent. Samples were analyzed using either SYBR green or using a probe-based approach. This finding nicely mirrors the finding made with T6, and suggests that altering this histidine residue may be universally beneficial for UvsX proteins used for RPA. Second, the utility of altering the nature of the very C-terminus of the protein was investigated. It was noted (see Figure 5) that Rb69 was very slightly shorter at the very C terminus relative to T6 and T4 UvsX. Examination of these proteins lead to the conclusion that the acidic residues found at the C terminus were loosely arranged in threes at the very protein terminus according to the rules (hydrophobic/structural)-(acidic)-(acidic). According to this model Rb69 was lacking one unit of this repeat relative to T4 and T6. It was hypothesized that the length of this acidic region would influence the RPA performance. To test this hypothesis, 2 novel clones with slightly extended the C-terminal Rb69 sequence were generated; in one case inserting the triplet of amino acids 'LSD' and in the second case inserting a tandem repeat of the triplet 'LDE' and thus 6 new residues (see Figure 20). The proteins containing these alterations were tested in assays using a probe-based detection approach. Although not every experiment gave completely consistent results (possibly in part because different start

copy numbers were used), in general a clear trend was noted. It was usually the case that the shape of the accumulation curve was slightly different between wild type Rb69, the 'LSD' mutant, and the '2xLDE' mutant. The mutants generally showed a very slightly later onset of detection, but then had a slightly sharper signal accumulation incline, and a slightly higher final total fluorescence (Figures 33 and 34). Although the extent of this effect was somewhat variable between different experiments performed under slightly variable conditions, it was nonetheless sufficiently clear to conclude that these alterations had significant biological effect. Without intending to be bound by any theory, these alterations may slightly reduce the affinity/stability of recombinase for certain substrates, particularly perhaps duplex DNA, and as such alter the reaction kinetics with a particular emphasis on reducing the late phase reaction slowing that is precipitated by the accumulation of product.

The next steps were to investigate the DNA binding loop2 sequences which were suspected of underpinning much variation. The Rb69 loop2 sequence NHT AMEIGGLYPKE IMG GG (SEQ ID NO:68) was substituted for the T6 loop NHT IETIEMFSKT VT GG (SEQ ID NO:69) except for the last variant threonine (bolded and underlined here) which was left as the native glycine found in Rb69. This was done because the T4 loop had a similar glycine to the Rb69 sequence, and assuming this residue was unimportant (or not strictly in the flexible loop region) it was left to avoid a more complex mutagenesis protocol. This new protein which had been generated in the backbone of the functional Rb69 H64S/2xLDE protein was tested. This protein was designated Rb69 H64S/T6-1/2xLDE in which T6-1 refers to the T6 DNA-binding loop2 lacking the last native threonine that precedes the pair of C-terminal glycines (see Figure 20 and legend). This protein was found to have no activity in RPA assays (Figure 39). It was speculated that this lack of activity might result from incompatibility between the DNA-binding loop and the residues in the nearby Walker motif. Rb69 has an unusual Walker motif in several respects. First, it does not have a serine but rather a threonine as the main putative catalytic residue of the motif in contrast to the other non-cyanophage proteins. This threonine is followed by another atypical residue, leucine, which is also not found in other UvsX proteins. In addition to this the glycine found at the beginning of the Walker A consensus is an alanine in Rb69 UvsX unlike any other UvsX molecule (apart from the near-identical JS98 protein) or even *E.coli* RecA.

In addition to the eccentric differences between Rb69 UvsX and other UvsX molecules, T6 UvsX also has eccentric residues in this region. In particular methionine 71 is

not found in most other UvsX proteins except those that are near-identical to T6, or phage 133 (see Figure 5). It was noted that phage 133 also had changes in the DNA-binding loop2 region (having leucines at the positions substituted to isoleucine in T6) which possibly represented evidence of a direct contact between these various residues. In all, the Rb69 Walker motif in its C-terminal region differs from T4 by 2-residues (compare Rb69 KTLFGL (SEQ ID NO:70) to T4 KSNFGL (SEQ ID NO:71)) and differs from T6 by 4 residues (compare Rb69 KTLFGL (SEQ ID NO:72) to KSNMSL (SEQ ID NO:73)). Changes in the Walker region in the backbone context of clone Rb69 H64S/2xLDE/T6-1 were generated making it like T4 (KSNFGL (SEQ ID NO:74)), like T6 (KSNMSL (SEQ ID NO:75)) or with changes made that are characteristic uniquely to T6 (KTLMSL (SEQ ID NO:76)). Attempts to express some of these clones failed despite the use of multiple sequenced clones apparently containing no errors. In fact it appeared that those clones that had been made equivalent to T4 or T6 sequences (KSNFGL (SEQ ID NO:77) or KSNMSL (SEQ ID NO:78)) would not express and purify properly. It was concluded that the 'SN' motif is not tolerated when the T6-1 DNA loop is inserted to replace the Rb69 DNA-binding loop2. This was most perplexing because this exchange is well-tolerated if the T4 DNA-binding loop 2 is used to replace Rb69, as later described. The one expressed clone (KTLMSL) appeared to have no activity in assays when tested.

A complete T6 DNA-binding loop2 sequence demonstrates activity

Clones were generated in which the last variant residue of the T6 DNA-binding loop 2 (NHT-IETIEMFSKT VMT GG (SEQ ID NO:79)) in the chimeric Rb69-T6 constructs were restored. Clones corresponding to Rb69 H64S/2xLDE/T6-1/ KSNMSL (SEQ ID NO:80) and Rb69 H64S/2xLDE/T6-1/ wtRb69 Walker were generated but with the repaired threonine and thus designated Rb69 H64S/2xLDE/T6/ KSNMSL (SEQ ID NO:81) and Rb69 H64S/2xLDE/T6/ wtRb69 Walker. Once again, the clone with an altered Walker motif would not express and purify. Without intending to be bound by any theory, this implies close biochemical context between these Walker A residues and the variant isoleucines present in the T6 DNA-binding loop2. However, a surprising discovery was that the latter clone possessing only a repaired T6 DNA-binding loop and no alterations to the native Rb69 Walker A motif did express and proved to be functionally active (Figure 46). Thus it appears that this last variant threonine residue is absolutely critical to the function of the T6 DNA-binding loop, at least when transferred to an Rb69 backbone. It was concluded that functional

chimeric proteins may be generated, and that all of the three substitutions between T4 and T6 DNA-binding loop 2 sequences have measurable functional implications.

Rb69 chimeras containing T4 DNA-binding loop2 sequences are active

Further chimeric molecules containing the DNA-binding loop2 sequence of T4 UvsX were generated. In contrast to the Rb69/T6 chimeras these proteins were active regardless of whether the Walker motif was left unaltered in the native state or changed to be T4-like (KSNFGL (SEQ ID NO:82)) even though such a Walker A motif was not tolerated when the T6 DNA-binding loop was employed. Again it is stressed that this could reflect direct contacts between the 'SN' motif and the first few residues of the DNA-binding loop2. Some tendency of the protein made with a native Rb69 Walker motif to precipitate more readily from concentrated stocks was observed, which could indicate a slight incompatibility between heterologous sequences, but this was only a slight effect.

Improved recombinase behaviour for Rb69 chimeras

From the above it may be concluded that DNA-binding loop 2 sequences may be exchanged between UvsX molecules from different origins to generate functional proteins in some cases. The Rb69 chimeric molecules generated were tested to determine whether they might display different characteristics to those exhibited by native Rb69. First, the protein was assayed to determine whether more resistant to overtitration of gp32 protein. Figure 43 shows that the delay in signal onset that is measured when mutant protein containing a T4 DNA-binding loop is used is decreased when higher quantities of gp32 are used than is the case with native Rb69. It was concluded that the engineered design contributed some of the more tolerant activity found in T4 and T6 UvsX proteins to the Rb69 chimera. Next the protein was assayed to determine whether T4 gp32 could be employed to replace Rb69 gp32, something that had not been possible with the native Rb69 protein. It was found that indeed amplification reactions could now be carried out using Rb69 protein containing the T4 DNA-binding loop (see Figure 44).

Thus it is possible to engineer UvsX proteins with novel biochemical activities by substituting key residues, and some of these are relatively improved compared to their native parents in RPA assays.

Other DNA-binding loop2 sequences

To extend this analysis further and to its logical conclusion Rb69 proteins containing all the various classes of DNA-binding loop2 sequences that were available were generated.

To ease this process a 'cassette' structure to the Rb69 clones were engineered, having a unique Bal I restriction enzyme site on one side and a KpnI restriction site on the other.

Synthetic oligonucleotides were cloned into Rb69 UvsX clones cut with these enzymes. The clones were generated as illustrated schematically in Figure 21. Problems were encountered when attempting to express some of these proteins. Purified protein for the RecA-substituted loop could not be recovered, and the KVP40-substituted loop aggregated during dialysis and could not be re-solubilised effectively afterwards. Of the remaining proteins, the Aeh1, Rb16/Aeh1 and Cyanophage-substituted loops were expressed well but had no activity in the assays. The phage 133-substituted loop did possess, albeit weak, activity in the assays.

Without intending to be bound by any theory, these clones were possibly slightly at a disadvantage relative to the studies done on T4 and T6 DNA-binding loops because in this case they were engineered into a wild-type Rb69 backbone rather than one containing H64S, and a more acidic C-terminus. No engineering of other parts of the Walker A motif were made either. Nevertheless the results provide a useful diagnostic on the likely tolerance of altered sequences in this region. First, it was noted that like T4 and T6, phage 133 DNA-binding loop could confer some activity to the hybrid protein. It can be concluded that to some extent there is a general tolerance to the short 'standard' loop lengths found in most sequenced phage UvsX molecules. Second, it was noted that Aeh1 failed, but this protein has a very unexpected mutation of the asparagine that begins the loop and is otherwise very highly conserved. It is anticipated that other substitutions would be necessary in order to tolerate this change. Finally, neither the cyanophage, nor the RecA loop appeared to be tolerated. In the case of the RecA loop this is not unexpected as this loop does not even conserve the loop length, being longer in RecA. Without intending to be bound by any theory, there may be problems for this protein to fold correctly, or it may tend to aggregate. The cyanophage loop is the same length as the parent Rb69 loop, however the sequence is almost completely different. As the cyanophage proteins are very diverged from Rb69, and have radically different Walker A motifs, it is expected that changing this loop in isolation will not suffice to generate a functional molecule.

T6 UvsX and derivatives exhibit UvsY-independent activity

An experiment was performed investigating the effects of modified DNA backbones in oligonucleotides used in RPA, in particular to assess whether they influenced a need for UvsY. In the course of this work it was observed that UvsY was not essential for the

amplification of DNA in experiments performed with T6 UvsX with the histidine 66 to serine mutation (T6 H66S). This unexpected phenomenon was further investigated, and the data described below confirmed that this property is substantially, although perhaps not entirely, attributable to the T6 origin of the recombinase species in the reaction.

5 Figure 52 illustrates an experiment performed to assess whether UvsY was required for amplification of DNA fragments from a template (generated by PCR) using a variety of primers. The experiment clearly indicated that for 3 of the 4 primer pairs used in this experiment (all combinations shared one common primer paired with an opposing primer a variable distance away in the template) products were generated in the absence of UvsY
10 which were of the expected molecular weight. A follow-up experiment is shown in Figure 53 in which the same template was employed, but some variable primer combinations were used (see legend). In this case 4 of the 5 combinations were successful regardless of the presence or absence of UvsY. Differences in product intensity were observed, and in some cases products were more abundant in the absence of UvsY. The results indicate UvsY is partially
15 dispensable in at least some amplification reactions performed with this recombinase (T6 H66S), SSB (Rb69 gp32), PEG 35,000 and polymerase (Sau Pol).

Investigations were extended to templates which were provided as complex genomic DNA. Of particular concern was that the extraordinary efficiency observed with the MS2
20 template might arise because this template had first been generated by PCR and might contain denatured or single-stranded templates. These situation could remove some 'constraints' placed on initiating RPA on true embedded sequences which are potentially difficult because of their tendency to form topologically strained structures during early cycles of amplification. The experiment shown in Figure 54 depicts the amplification of DNA from human genomic DNA using pairs of primers (one common primer) which generate
25 progressively larger fragments. In this case the results were rather more variable than observed with the MS2 template. However, at least two of the combinations generated fragments that were considered to be the expected length even when UvsY was omitted (all reactions functioned in the presence of UvsY). This work was extended in the experiment shown in Figure 55. Once again, in some cases, DNA products of the expected sizes were
30 generated in paired reactions even when UvsY was omitted, and once again there was significant variability on the outcome depending on the primer pairs and/or anticipated product size. It was believed that reactions 2 and 4 were successful in both cases.

Another set of experiments were performed to assess whether this remarkable and previously unnoticed activity, believed to be attributable to of T6 derivative recombinase, (and possibly associated factors used here) extended to a difference in requirement for polyethylene glycol. Figure 56 shows that despite a partial resistance to the need for UvsY, the omission of PEG results in the absence of significant DNA synthesis. It was concluded that PEG was still required to achieve useful DNA amplification from low target concentration samples.

Next assessed was whether the type of gp32 employed affected the UvsY-independent nature of these amplification reactions. Figure 57 shows the results of an experiment in which T4 gp32 is employed instead of Rb69 gp32. As shown in Figure 57, DNA was still amplified in the presence of T4 gp32, albeit with slightly different ratios of products. Figure 58 extends this work and shows that DNA is still synthesized in a heterologous system employing Aeh1 gp32, although no products of the expected size were generated in the absence of UvsY. Note however that DNA of some description was made in the absence of UvsY which was consistent with a significant biochemical difference between these reactions and earlier reactions using all T4 reagents. In the models described herein, to synthesise/amplify any DNA visible on gels at endpoint a minimum number of loaded recombinase filaments are required, which were considered to be too few in the absence of UvsY acting as a re-loading/stabilizing agent. Thus, it was concluded that exchanging gp32 species does influence the efficacy of reactions under these conditions, but that in all cases DNA synthesis does occur even in the absence of UvsY in contrast to earlier results attained with T4 reagents. It was concluded that the T6-derivative UvsX is primarily responsible for permitting high-loading of recombinase filaments in contrast to the situation with T4 UvsX. This presumably could reflect difference in the DNA-binding domains as well as inter-subunit surfaces involved in stabilizing the co-operative filament structure.

This difference in UvsX behaviour was further confirmed in the experiment shown in Figure 59, showing a complete absence of DNA synthesis when T4 UvsX is substituted for T6 H66S.UvsX, and then UvsY is omitted. Similar results were obtained in the experiment shown in Figure 60 in which a similar experiment is performed but using T4 gp32 throughout – T4 UvsX absolutely requires UvsY while T6 H66S does not in these experiments. A kinetic experiment is shown in Figure 61. As shown in Figure 61, detection kinetics are moderately similar between T4 and T6 H66S experiments. However when UvsY is omitted there is little

consequence for the T6 H66S amplification kinetics, while the T4 recombinase shows no activity. In other experiments with other templates, an obligate need for UvsY even when using T6 H66S, was noted. Thus it was concluded that UvsY is only partially dispensable when using this recombinase, and it can still improve reaction behaviour and play a role in robust and consistent RPA behaviour between targets.

Next, investigations into whether this unusual property was observed with unmodified T6 UvsX, and whether it extended to other recombinases (such as Rb69 UvsX and Aeh1 UvsX) were performed. Figure 62 shows very clearly that DNA is efficiently synthesized with at least one oligonucleotide combination when T6 recombinase is employed in the absence of UvsY. It was concluded that the unusual property of UvsY-independence not observed with T4 UvsX extends to the unmodified T6 UvsX, albeit there were differences in product accumulation levels between T6 UvsX and T6 H66S UvsX confirming their biochemical distinction.

Figure 63 shows that results of an experiment to determine whether Rb69 UvsX could operate in the absence of UvsY. While caution is advised on interpretation of the results because one of the amplicons did not amplify even with UvsY, the principle observation was the lack of DNA generated when UvsY was omitted. Without intending to be bound by any theory, this implies that, like T4 UvsX, Rb69 UvsX cannot readily support efficient amplification without the presence of UvsY. Figure 64 extends this analysis to the employment of phage Aeh1 components. As shown in Figure 64, amplification is efficient in a heterologous system comprising Aeh1 UvsX, Aeh1 UvsY and Rb69 gp32, however if Aeh1 UvsY is omitted no amplification is seen. Next, the activity of a modified Rb69 UvsX containing, amongst other things, the DNA binding loop2 sequence of T6, was assessed. This experiment was performed to assess whether the activity of T6 derivatives might arise from the distinct T6 DNA binding loop2 sequence. In this case, no amplification in the absence of UvsY was observed, although caution is advised as amplification seemed rather weak in the presence of UvsY. However, taken at face value, this result does not support that the T6 DNA binding loop 2 is wholly responsible for the unusual behavior of T6 UvsX and its derivatives, or that this property cannot be trivially transferred in isolation.

These results collectively show that T6 UvsX and its derivatives are unusual insofar as when co-incubated in the presence of gp32 species of various types (T4, Rb69 and Aeh1) it is capable of supporting significant recombination activity without a need for UvsY.

Without intending to be bound by any theory, existing models suggest that a limiting component of recombinase-driven amplification systems is the concentration of recombinase-loaded filaments. These are not considered to be abundant when T4 UvsX is co-incubated in the presence of T4 gp32, and in the absence of UvsY and crowding agents. However the evidence suggests that for T6 UvsX this competitive environment is perhaps shifted in the favor of recombinase, so much so that UvsY can be dispensed with in some cases. For this to occur, it could be inferred that T6 UvsX may have a higher affinity for single-stranded DNA than T4 UvsX, and/or that it is less likely to disassemble from filaments as a consequence of active ATP hydrolysis. In turn these properties could arise due to higher affinity of the DNA binding elements of the recombinase for nucleic acids, and/or via higher affinity between protein subunits in the filaments leading to a reduction in disassembly behaviour. However, it is noteworthy that reactions appeared more robust on the whole when UvsY was included. Occasionally, in its absence, DNA was synthesized but products of the expected size did not accumulate. This outcome could reflect an abundance of active filaments but some other fundamental flaw in the RPA reaction cycle.

Without intending to be bound by any theory, two possible mechanisms to explain why UvsY enhances RPA functionality even when it is not strictly required for some amplification activity are proposed herein. First, UvsY could confer full and even loading of filaments on oligonucleotides ensuring that they are coated to their 5' ends, and undergo efficient recombination along their length. In the absence of UvsY, according to this rationale, filaments may only be partially loaded and this could lead to a situation in which recombination leads to constrained intermediates (no free unwinding possible at the substrate 5' ends) most of the time which are unstable and lead to disassembly of recombinase/synthesis intermediates before complete synthesis along a target has occurred. This could favor very short products such as primer dimers that require little processive DNA synthesis. A second alternative is that UvsY plays an active role in the DNA synthesis process as it is ongoing. For example, UvsY could promote recombinase-loading of the outgoing strand and re-invasion to cause a 'bubble migration' activity. Such bubble migration synthesis could act to decrease topological strain on the synthetic complex. Similarly, the processivity of elongation complex might rely on accessing the 3' end of DNA which is still partly coated with UvsX, and this might require UvsY presence. In any case, the data support the notion that UvsY may play a role in the RPA process that is more sophisticated than

simply increasing the steady state number of recombinationally active filaments in the reaction environment.

Furthermore the use of different gp32 species may influence the UvsY-dependence of RPA reactions. Experimental data provided here, including competition oligonucleotide competition data and thermal stability data presented below, suggest that T4 gp32 may have a particularly high affinity for DNA when compared to Rb69 gp32 and Aeh1 gp32. Thus, according to a model in which UvsX and gp32 compete for common substrates as described earlier, it may be beneficial for the recombinase if a gp32 with a lower DNA affinity is employed. Thus Rb69 gp32 is likely to favor recombinase-loading in such a competitive environment.

Manganese can support RPA reactions

Manganese can replace magnesium ions to support DNA amplification by the RPA system. In particular the useful range of manganese ions for supporting robust amplification is significantly lower than that found for magnesium. The most effective amplification occurs when manganese is present at roughly 1 to 3 mM (Figure 47). Higher concentrations are progressively inhibitory to significant product accumulation. These low levels of supporting ion are something of a surprise as in some cases this is an insufficient quantity to saturate the abundant ATP and dNTP species in the reaction (ATP is used at 3mM).

Heparin can act as a noise-suppressing reagent

The effects of heparin on RPA reactions were investigated. This was in part in an effort to establish the resistance of RPA reactions to agents commonly found in clinical and environmental samples. It was surprising to discover that RPA was rather resistant to the inclusion of heparin in the amplification reactions. Indeed it even appeared that heparin could improve the outcome of RPA reactions, apparently by reducing the rate at which primer artefacts accumulate in RPA reactions. Figure 49 reveals how the inclusion of heparin at 20ng/μl results in a delay in the accumulation of primer artefacts which appear if RPA is permitted to run without a target present in the reaction. Using a probe-based sensing approach, inclusion of heparin in RPA was tested to determine whether it would improve the behaviour of RPA reactions. Figure 50 explores the effects of including heparin in amplification reactions. The following phenomena are observed: the time of onset of signal detection are similar regardless of the presence of heparin, however when present heparin leads to more consistent time of onset of detection at low copy numbers. Heparin slightly

decreases the total signal which develops in the reaction. It was concluded that probably heparin acts as a 'sink' for UvsX or other DNA binding proteins and can help to buffer it from excessive activity which may benefit noise rather than signal under certain circumstances.

5 ***E. coli* exonuclease III can function as a primer polishing agent in RPA**

E. coli endonuclease IV (Nfo) or *E. coli* exonuclease III were included in RPA reactions that include proprietary fluorescent probe sensing system (Piepenburg et al., 2006) as an agent to process abasic-site containing probes. However during investigations into novel probe structures some surprising and unexpected observations were made, namely that
10 supposedly 3'-blocked primers could be efficient amplification primers when used in reactions containing exonuclease III, and perhaps to a lesser extent if containing endonuclease IV (Nfo) (see Figure 51). It was hypothesized that blocked primers employed in these cases were being unblocked by the activity of the enzymes. Both of these enzymes have reported activities which include 3'-exonuclease activity as well as having 3'-diesterase or
15 phosphatase activities. Without intending to be bound by any theory, it is likely they either 'polish' the blocking group from the final base, or remove the final base with the blocking group on it. It is not possible to distinguish between these possibilities from these experiments. However the potential ability to 'unblock' primers in a sequence-dependent manner has certain potentially useful applications.

20 ***S. aureus* Pol I large fragment is functional in RPA reactions**

RPA works efficiently with Bsu polymerase as previously shown (See Piepenburg et al. U.S.S.N. 10/931,916). It has also been shown to function with the Klenow fragment of *E. coli* Pol I, and with Bst polymerase. Other polymerases were examined in attempts to extend the breadth of polymerases that may be used in RPA reactions. The polymerases
25 examined included repair class polymerases, and polymerases which lack proof-reading activity. The large fragment of such polymerases, as opposed to the full protein, were also examined. A sequence corresponding to the *S. aureus* Pol I was identified in the Genbank entry locus BX571857 which is the genome sequence of methicillin-sensitive *S. aureus* strain MSSA476. The complete polymerase coding sequence corresponds to the complement to
30 positions 1740769 to 1743399 of the genomic sequence and the putative encoded polypeptide has the TrEMBL accession number Q6G8N6. A fragment of this coding region was amplified from MSSA476 genomic DNA corresponding to position 865 to 2631 of the coding region,

thus omitting the first 288 amino acid residues which correspond principally to the 5'-3' exonuclease domain. This fragment was cloned into pET21+ and included a hexahistidine-encoding tag incorporated into the PCR primers at the 5' end. This protein expressed efficiently and was readily purified on Ni-NTA agarose. This protein was tested in RPA reactions as shown in Figure 48. It was observed that the *S.aureus* enzyme (referred to as Sau polymerase) works very well and seems at least as efficient as the Bsu polymerase.

gp32 Activity

As demonstrated below, novel activity assays for gp32 proteins demonstrate their distinct biochemical activities. gp32 proteins were derived from several different bacteriophages. In one experiment, gp32 activity was assessed by establishing a reaction environment in which the mass of gp32 contained in the reaction was titrated until it was just limiting in activity as assessed by a nuclease-protection assay. Figure 66 illustrates such assay, which was performed to determine the quantity (mass) of Rb69 gp32 required to inhibit the cutting of a reporter probe oligonucleotide by the endonuclease IV (Nfo) of *E.coli*. In this assay, cutting was monitored by rising fluorescence which occurs as a consequence of nucleolytic attack on a tetrahydrofuran (abasic mimic) positioned between a fluorophore and dark quencher in the probe. In the absence of gp32 the probe was cut so rapidly that by the time the tube was transferred to the fluorometer for measurement it was already almost completely degraded (high fluorescence). Conversely, when 250 ng/ μ L of Rb69 gp32 was included in the reaction, cutting was completely abolished and a flat line resulted throughout the assay time (100 seconds). Intermediary quantities of the gp32 protein resulted in fluorescence increase curves of various slopes consistent with a strict relationship between protein mass and protective capacity. The results demonstrate the utility of this assay in establishing the 'activity' of a gp32 preparation.

As shown in Figure 66, it is possible to establish a ratio of probe oligonucleotide and gp32 protein that is on the boundary of complete protection, such as between 83 and 100 ng/ μ L. At this concentration of gp32 cutting occurred, but only slowly, and any changes in gp32 activity were likely to be easily observed by difference in cutting rate. At such a concentration, the reaction was challenged with additional added reagents or changes in environmental conditions, such as temperature, and the efficacy of gp32 in probe protection was assessed. Figure 67 shows the results of an experiment in which the consequences of challenging the reaction with additional single-stranded or double-stranded DNA were

assessed. In this experiment, the effects of these challenges on Rb69 gp32, T4 gp32 and Aeh1 gp32 were compared. In all cases challenge with competitor ssDNA at a defined time resulted in a sharp increase in probe attack.

The results demonstrate that the distribution of gp32 must be highly dynamic, supporting the notion that both association and dissociation events occur frequently in RPA reactions (although in the presence of crowding agents and other RPA reagents the kinetics may be altered). While this competitive effect of ssDNA was strong and similar between different gp32 species, significant differences were noted when the system was challenged with double-stranded DNA. When challenged with 10 times the mass of dsDNA (compared to probe) Aeh1 and RB69 gp32 showed only very slight increases in cutting activity. In contrast T4 gp32 showed a very significant increase in cutting activity. While not intending to be bound by any theory, the results suggest that the relative affinities of the gp32 species to double-stranded DNA were significantly variant. These results further suggest that there are could be significant differences in the late RPA reaction behaviour depending on the species of gp32. Rb69 or Aeh1 gp32 are likely to be more strongly partitioned between single-stranded and double-stranded DNA, while T4 gp32 is likely to be titrated out onto the duplex products. This may account for some of the improved activity noted with Rb69 gp32 in some RPA reactions. It is possible that T4 gp32 simply has a higher overall DNA affinity, which would be consistent with the results of the next experiment detailed below.

In another variation of the probe protection assay the effects of temperature on the activity of the gp32 in protecting the probe were investigated. Figure 68 shows the effects of progressively increasing the temperature of the reaction environment over time and reveals that at a certain point the protective properties of the gp32 suddenly decrease. This presumably represents the upper temperature at which the protein functions efficiently. It was noted that the profiles are markedly different between the 3 species tested here. Aeh1 gp32 became less effective above about 40 degrees centigrade and losing protective capacity very quickly above this temperature. By 42 degrees it lost almost all of its activity. In contrast Rb69 gp32 retains full activity up until about 42 degrees and then slowly starts to lose activity. While compromised, it still affords some protective capacity up until 47 degrees in this assay. The most powerful protective capacity was, however, observed for T4 gp32 which only started to show a slight decrease in effectiveness at 49 degrees, the highest temperature assayed in this experiment. Thus was deduced that the operational temperature range for

these 3 proteins is clearly and measurably distinct. This should have some considerable significance when deciding which gp32 species is most suitable for a given application, and may reflect both the thermal stability of the protein itself as well as the relative DNA binding affinity of the protein.

5 It is understood that "acid C-term," acidic C terminus, acid N-term, and acidic N terminus refer to the optional addition of one or more acid amino acids, such as (LDE)_n or (LSD)_n where n = 1 to 4 or 10 or fewer acidic amino acids to the C or N terminus of the protein. In addition, any of the proteins described anywhere in this specification, including the recombinase (e.g., UvsX), recombinase loading agent (e.g., UvsY), and single stranded
10 binding protein (e.g., gp32) may optionally include a His tag at the N terminus, at the C terminus, or between the N terminus and C terminus of the protein in addition to any other modification (such as acidic C or N terminus). His tag is understood to mean 10 or fewer amino acids comprising Histidines in series or Histidine and Glutamine (HQ, or QH) in series - in a preferred embodiment, the number is 6. Furthermore, His tags may also refer to amino
15 acids such as HQHQHQHQHQ (SEQ ID NO:83) which is less than 10 amino acids in length such as HQHQHQ (SEQ ID NO:84). For example, if a protein has both an acidic C terminus and a C-terminus histidine tag, the protein may have a configuration such as [protein]-[acidic residues]-[histidine tag] or such as [protein]-[histidine tag]-[acidic residues]. Alternatively, a protein with both an acidic N terminus and a N terminus histidine tag may have a
20 configuration of [acidic residues]-[histidine tag]-[protein] or such as [histidine tag]-[acidic residues]-[protein].

EXAMPLES

The invention, having been generally described, may be more readily understood by reference to the following examples, which are included merely for purposes of illustration of
25 certain aspects and embodiments of the present invention, and are not intended to limit the invention in any way. Other aspects, advantages, and modifications are within the scope of the following claims.

EXAMPLE 1: Cloning and protein expression

All DNA manipulations were performed using standard techniques, in particular
30 cloning using PCR, PCR-based mutagenesis procedures, and standard restriction digestion and ligation. Sequencing was performed by Lark technologies Ltd, Saffron Walden, UK. All

proteins were expressed in *E.coli* and purified in 1M NaCl following lysis using lysozyme at 1mg/ml and 2-3 freeze thaw cycles. Ni-NTA resin was purchased from Qiagen.

Amplification reactions

The conditions for individual amplification reactions are described in the detailed descriptions provided below. In general reactions were monitored in real-time either by the inclusion of SYBR green dye, or more often by employment of a probe-based approach developed by us (see Piepenburg et al. 2006). In this case the probe is a third DNA primer which contains an internal tetrahydrofuran residue (abasic site mimic) flanked by a fluorophore and a quencher. On hybridization to amplified DNA this probe becomes a substrate for the endonucleolytic activity of endonuclease IV (Nfo) or exonuclease III which are enzymes included in the reaction.

The sequence of fluorescent probes described here are as follows:

- SATamra1 5'-tgtaattgaacaagtgtacagagcatt(T)a(H)ga(q1)tatgcgtggag-Biotin-3' (SEQ ID NO:85)
- 15 SATamra2 5'-tgtaattgagcaagtgtatagagcatt(T)a(H)ga(q2)tatgcgtggag-Biotin-3' (SEQ ID NO:86)
- BsFlc 5'-catgattggatgaataagctgcagc(F)g(H)t(q3)aaaggaaacta-Biotin-3' (SEQ ID NO:87)

Where (T) is dT-TAMRA, (F) is dT-Fluorescein, (H) is THF, (q1) is dT-BHQ1, (q2) is dT-BHQ2, (q3) is dT-DDQ1. Nfo enzyme was used at 200 ng/μl, but almost all probe-based experiments employed exonuclease III at 65 ng/μl. Excitation/detection was at 485/525 nm (SYBR green or probe BsFlc) or 530/575nm (SATamra1/2). Measurements were taken every 30 or 45 seconds. Fluorescence probe data was normalised against water controls, and the pre-amplification baseline was adjusted. In general the logarithm of the normalised fluorescence read-out was plotted against time for the probe-based experiments.

25 Amplification primers:

Bacillus subtilis:

J1 – 5'-acggcattaacaaacgaactgattcatctgcttg (SEQ ID NO:88)

K2 – 5'-ccctaatttctccgagaacttcattcaagcgtc (SEQ ID NO:89)

MRSA:

30 sccIII – 5'-ccaatatttcatatatgttaattcctccacatctca (SEQ ID NO:90)

orf45a (aka orfX) – 5'-cccaaggcgaaagcgactttgtattcgtcattggcgatcaaacg (SEQ ID NO:91)

sccII-35 IV – 5' - ctcaaagctagaactttgcttcactataagtattc (SEQ ID NO:92)

MS2:

MS2 down RT2 – 5' - cttagtaagcaattgctgtaaagtcgtcac (SEQ ID NO:93)

MS2 down 5 – 5' - ccagtagcgacagaagcaattgattggttaaatt (SEQ ID NO:94)

5 MS2 up 2 – 5' - ttccgactgcgagcttattgtaaggcaatg (SEQ ID NO:95)

MS2 up 4 – 5' - cctcgcgatctttctctcgaaattaccaatca (SEQ ID NO:96)

MS2 up5 – 5' - ccatgtcgaagacaacaaagaagtcaactctt (SEQ ID NO:97)

MS2 up 6 – 5' - catctactaataagacgccggccattcaaacatg (SEQ ID NO:98)

MS2 up 7 – 5' - cccgattccctcagcaatcgagcaaaactccgg (SEQ ID NO:99)

10 *Apolipoprotein B:*

ApoB4 – 5' - cagtgtatctggaaagcctacaggacacacaaaa (SEQ ID NO:100)

ApoB300 – 5' - tgctttcatacgttttagcccaatcttgatag (SEQ ID NO:101)

ApoB3 – 5' - tgacaagtgtgctataaacctggcctaccagag (SEQ ID NO:102)

ApoB7 – 5' - ttgatacattcggctcgtgtatctctata (SEQ ID NO:103)

15 ApoB10 – 5' - gatacattcggctcgtgtatctctagg (SEQ ID NO:104)

Clones were constructed by PCR using genomic DNA of T6 phage, Rb69 phage, Aeh1 phage, or phage KVP40. Figure 1 shows the schematic layout of novel clones encoding diverse recombination machinery from the myoviridae. A modified pET21+ plasmid (Novagen) was used, and hexahistidine tags were engineered into the PCR primers to encode in-frame tags at either the N terminus (UvsY proteins) or at the C terminus (UvsX and gp32 proteins). In alignments and discussions later the amino acid residue numbers refer to the position in the native proteins as documented in the relevant databases. In the case of UvsY there will be 6 histidines and a methionine preceding this in the clones used.

EXAMPLE 2: Primary Sequence Alignment of Diverse Recombinase Proteins

25 *Primary Sequence Alignment of T4 UvsX and E.coli RecA*

The web-based tool MAFFT (accessed via the ExPASy proteomics server) was used to align the primary polypeptide sequences of T4 UvsX and *E.coli* RecA, as shown in Figure 2. This alignment was consistent with those generated and discussed elsewhere. Based on the known crystal structure of *E.coli* RecA the position of three regions of interest namely the Walker A motif involved in ATP binding and hydrolysis, the mobile DNA binding loop 1, and the mobile DNA binding loop 2 sequences are boxed. Under the alignment symbols

indicate amino acid identity between all homologs (*), conserved substitutions (:), or semi-conserved substitutions (.).

Model of RecA structure with superimposition and labelling of equivalent T4 UvsX residues

A model of the RecA nucleoprotein filament was generated using CN3D and a dataset
 5 downloaded from the NCBI database, PDB entry 1N03 (associated citation Vanloock MS et al., Structure 2003 Feb;1(2):187-96). Using the alignment in Figure 2 the putative position of T4 UvsX residues was mapped to this RecA structure as an exercise in providing insight into the possible position of UvsX amino acids of interest and their proximity to one another. Figure 3 shows the model of RecA structure with superimposition and labelling of equivalent
 10 T4 UvsX residues based on primary sequence alignment. Figure 3A shows the screenshot looking down the axis of the model RecA filament with the central hole being the approximate location of bound DNA. The approximate location of the Walker A motif and mobile DNA binding loops is indicated for a single subunit and is on the surface facing the nucleic acid. Figures 3B and 3C show two zoomed shots are taken of the region to which
 15 ATP is bound on the surface indicated in (A). the putative positions of T4 UvsX residues G60, S64, S67, F69, G70, H195, and M208 are indicated in Figure 3. Also indicated are the approximate locations of the beginning and end of mobile DNA-binding loop 2. That these amino acids are positioned exactly as shown in this model is unlikely given the significant divergence between RecA and UvsX, however these approximations are probably of
 20 meaningful utility for the study herein.

Primary sequence alignment of T4 and T6 gp32 and UvsY proteins

The web-based tool MAFFT (accessed via the ExPASy proteomics server) was used to align the primary polypeptide sequences of T4 and T6 gp32 and UvsY proteins, as shown in
 25 Figure 4. This alignment revealed only small differences between these proteins. The UvsY proteins had only 2 highly conservative substitutions. Under the alignment symbols indicate amino acid identity between all homologs (*), conserved substitutions (:), or semi-conserved substitutions (.).

Primary sequence alignment of diverse UvsX proteins

30 The web-based tool MAFFT (accessed via the ExPASy proteomics server) was used to align the primary polypeptide sequences of T4, T6, phage 133, Rb69, Aeh1, Ae65, KVP40, Rb43, PSSM2, and PSSM4 UvsX proteins, as shown in Figure 5. Several regions of interest were boxed, namely the Walker A motif (or 'P-loop') involved in DNA binding and

hydrolysis, the mobile DNA binding loop 1, and the mobile DNA binding loop 2. Certain residues under discussion have been highlighted. All amino acid differences between T4 and T6 UvsX are shown in bold. Under the alignment symbols indicate amino acid identity between all homologs (*), conserved substitutions (:), or semi-conserved substitutions (.).

5 *Primary sequence alignment of diverse UvsY proteins*

The web-based tool MAFFT (accessed via the Expasy proteomics server) was used to align the primary polypeptide sequences of T4, T6, phage 133, Rb69, Aeh1, KVP40, Rb43, PSSM2, and PSSM4 UvsX proteins, as shown in Figure 6. In this alignment the PSSM4 sequence was derived from our own translation of the genomic DNA, the NCBI entry
10 apparently erroneously omitting the first 43 residues from the polypeptide sequence. Under the alignment symbols indicate amino acid identity between all homologs (*), conserved substitutions (:), or semi-conserved substitutions (.).

Primary sequence alignment of diverse gp32 proteins

The web-based tool MAFFT (accessed via the Expasy proteomics server) was used to
15 align the primary polypeptide sequences of T4, T6, Rb69, Aeh1, KVP40, Rb43, PSSM2, and PSSM4 gp32 proteins, as shown in Figure 7. In this alignment the PSSM2 sequence was derived from our own translation of the genomic DNA, the NCBI entry apparently erroneously omitting the first 25 residues from the polypeptide sequence. Under the alignment symbols indicate amino acid identity between all homologs (*), conserved
20 substitutions (:), or semi-conserved substitutions (.). Also indicated by arrows are the positions of residues implicated in the co-ordination of zinc in T4 gp32. Also indicated by a line above the sequence is a common sequence, FKRK (or FKRQ in Rb43) which is absent in cyanophage gp32 proteins, and is implicated in co-operative binding as is the zinc atom of T4 gp32. Lack of co-ordinating residues in cyanophage gp32 proteins suggests that these
25 proteins may not require metals such as zinc, cobalt, nickel etc. for activity. The re-organised status of the KVP40 metal-binding region suggests that this protein may not bind zinc, but rather a different metal atom, or that it may show altered requirements for zinc during growth, or altered sensitivity to replacement assault by competitor metal atoms.

30 EXAMPLE 3: T6 UvsX substituted for T4 UvsX in RPA reactions using heterologous components

RPA reactions were configured using primers Rs8179145-2 and Rs8179145-3 whose sequences are indicated. Target DNA was human genomic DNA, and reaction conditions

were as follows: 100 mM potassium acetate, 50 mM Tris Acetate pH 8.3, 50 mM phosphocreatine, 3 mM ATP, 200 μ M dNTPs, 300 nM Rs8179145-2 primer, 300 nM Rs8179145-3 primer, 150 ng/ μ L T4 or T6 UvsX, 1000 ng/ μ L T4 gp32, 40 ng/ μ L T4 UvsY, 42 copies of human genomic DNA, 5% Carbowax 20 M, and 32 ng/ μ L Bsu polymerase. After 90 minutes samples were purified via centrifugation through a Qiagen PCR product clean-up column. Purified samples were analyzed on an ethidium bromide stained agarose gel. The expected amplicon size from the human locus Rs817945 was 205 bp. Asterisks on the gel shown in Figure 8 indicate the position of the expected band, 205 bp and the position of marker bands is indicated on the left. As shown in Figure 8, T6 UvsX can effectively be substituted for T4 UvsX in RPA reactions using heterologous components.

RPA reactions were established to compare the kinetics of T6 and T4 UvsX using SYBR green dye, using primers J1 and K2 under the following conditions: 50mM Tris.acetate pH 7.9, 100 mM Potassium acetate, 14mM Magnesium acetate, 50mM Creatine phosphate (Calbiochem), 3mM ATP (Roche), 200 micromolar dNTPs, 50 ng/ μ l creatine kinase (Roche), 120 ng/ μ l UvsX of T4 or T6, 30ng/ μ l UvsY, 900ng/ μ l gp32, 30 ng/ μ l Bsu polymerase, 5% Carbowax 20M, 300nM amplification primers, 1:50,000 dilution from stock of SYBR green (Invitrogen). Reactions were established on ice in a 96-well plate, and then transferred to a BIOTEK Flx-800 fluorescence microplate reader with stage set to 38°C at which time measurements were taken periodically from a top-reading probe. Samples contained either no target (water) or 50 or 5000 copies of *B.subtilis* genomic DNA containing the target sequence. Samples contained either T4 or T6 UvsX, and the recombinase and presence of target is shown in the legend. Each sample was run in duplicate.

Positive signals developed in all samples during the 60 minute incubation, and the time of signal increase was earlier in the target-containing samples than in non-target samples as expected. As shown in Figure 9, the time at which signal increase was first detected was similar between T4 and T6 samples. However the curves developed with different slopes and final maxima. T6 gave less sharp signal accumulation and less high final signals.

RPA reactions were also established to compare the kinetics of T6 and T4 UvsX using fluorescent probe, using primers orfx45a (120nM) and scii35IV(480nM) under the following conditions: 50mM Tris.acetate pH 7.9, 100 mM Potassium acetate, 14mM Magnesium acetate, 50mM Creatine phosphate (Calbiochem), 3mM ATP (Roche), 200 micromolar dNTPs, 50 ng/ μ l creatine kinase (Roche), 120 ng/ μ l UvsX of T4 or T6, 30ng/ μ l

UvsY, 900ng/μl gp32, 50 ng/μl Bsu polymerase, 5% Carbowax 20M, 120nM fluorescent probe SATamra2. Exonuclease III was included at 65ng/μl. Reactions were established on ice in a 384-well plate, and then transferred to a BIOTEK Flx-800 fluorescence microplate reader with stage set to 38°C at which time measurements were taken periodically from a bottom-
 5 reading probe. Samples contained either no target (water) 100, or 1000 copies of MRSA 3 (mecI) genomic DNA containing the target sequence. Samples contained either T4 or T6 UvsX, and the recombinase and presence of target is shown in the legend. Each sample was run in duplicate.

Positive signals developed in the template positive samples during the 90 minute
 10 incubation, and the time of signal increase was earliest in the highest target-containing samples. As shown in Figure 10, the time at which signal increase was first detected was similar between T4 and T6 samples, particularly for the 1000 copies samples, however the curves developed with different slopes and final maxima. T6 gave less sharp signal accumulation and less high final signals.

15 **EXAMPLE 4: Engineered T6 UvsX protein constructs**

The parent plasmid clone containing T6 UvsX in a modified pET21+ vector was altered using standard PCR mutagenesis protocols. A schematic layout of the relation of the coding region/primary polypeptide sequence to putative structural elements is shown at the top of Figure 11. Modifications were made to three regions which are shown as boxes on the
 20 schematic, the Walker A motif, the DNA binding loop1 and, DNA binding loop2. Several regions and amino acids were targeted and these are indicated on the lower schematics next to the name given to the clone. Numbers refer to the position of the amino acid in the wild type T6 UvsX protein, hence H66S means that the histidine present as amino acid 66 in wild type T6 was altered to a serine. On the left of the Figure 11, a simple representation of the
 25 general activity of the protein produced for this clone when tested in RPA assays is shown.

Comparison of T6 UvsX H66S and wild type T6 UvsX

RPA reactions were established to compare T6 UvsX H66S and wild type T6 UvsX using primers J1 (120nM) and K2 (480nM) under the following conditions: 50mM Tris.acetate pH 7.9, 100 mM Potassium acetate, 14mM Magnesium acetate, 50mM Creatine
 30 phosphate (Calbiochem), 3mM ATP (Roche), 200 micromolar dNTPs, 50 ng/μl creatine kinase (Roche), 120 ng/μl UvsX of T4 or T6 UvsX H66S, 45ng/μl T4 UvsY, 900ng/μl T4 gp32, 30 ng/μl Bsu polymerase, 5% Carbowax 20M, 120nM fluorescent probe BsFlc.

Exonuclease III was included at 65ng/μl. Reactions were established on ice in a 384-well plate, and then transferred to a BIOTEK Flx-800 fluorescence microplate reader with stage set to 38°C at which time measurements were taken periodically from a bottom-reading probe. Samples contained either 100, or 1000 copies of *B.subtilis* genomic DNA containing the target sequence. Samples contained either T4 or T6 UvsX H66S, and the recombinase and presence of target is shown in the legend in Figure 12. Each sample was run in duplicate.

The sequence of T6 UvsX H66S is as follows: MSIADLKSRL IKASTSKMTA
ELTTSKFFNE KDVIRTKIPM LNAISGAID GGMQSGLTIF AGPSKSFKSN
MSLTMVAAYL NKYPDAVCLF YDSEFGITPA YLRSMGVDPE RVIHTPIQSV
10 EQLKIDMVNQ LEAIERGEKV IVFIDSIGNM ASKKETEDAL NEKSVADMTR
AKSLKSLFRI VTPYFSIKNI PCVAVNHTIE TIEMFSKTVM TGGTGVMYSA
DTVFIIGKRQ IKDGS DLQGY QFVLNVEKSR TVKEKSKFFI DVKFDGGIDP
YSGLLDMALE LGFVVKPKNG WYAREFLDEE TGEMIREEKS WRAKDTNCTT
FWGPLFKHQP FRDAIKRAYQ LGAIDSNEIV EAEVDELINS KVEKFKSPES
15 KSKSAADLET DLEQLSDMEE FNE (SEQ ID NO:105).

As shown in Figure 12, positive signals developed in the samples during the 90 minute incubation, and the time of signal increase was earliest in the highest target-containing samples. Signals developed earlier in the T6 UvsX H66S – containing samples, particularly for the 1000 copies samples, and the curves developed higher final maxima. Based on this study, it was concluded that *T6 UvsX H66S* performs better in these assays than wild type T6 UvsX. However the slope of the signal accumulation using this system was similar between the 2 proteins, and therefore it is unlikely that T6 UvsX H66S exactly reproduces the activity of T4 UvsX in this assay.

Kinetic behaviour of other mutants of T6 UvsX

25 RPA reactions were established using mutant T6 UvsX components, using primers J1 (120nM) and K2 (480nM) under the following conditions: 50mM Tris.acetate pH 7.9, 100 mM Potassium acetate, 14mM Magnesium acetate, 50mM Creatine phosphate (Calbiochem), 3mM ATP (Roche), 200 micromolar dNTPs, 50 ng/μl creatine kinase (Roche), 120 ng/μl UvsX of T6 or T6 UvsX H66T or T6 UvsX M71F/S72G or T6 UvsX S164V/A166S, 45ng/μl
30 T4 UvsY, 1000ng/μl T4 gp32, 30 ng/μl Bsu polymerase, 6% Carbowax 20M, 120nM fluorescent probe BsFlc. Exonuclease III was included at 65ng/μl. Reactions were established on ice in a 384-well plate, and then transferred to a BIOTEK Flx-800 fluorescence microplate

reader with stage set to 38°C at which time measurements were taken periodically from a bottom-reading probe. Samples contained either water or 200 copies of *B.subtilis* genomic DNA containing the target sequence as indicated in the legend.

As shown in Figure 13, positive signals developed in some samples during the 90 minute incubation. Signals developed earliest in the T6 UvsX S164V/A166S and then wild-type samples. Signal accumulated much later in the T6 UvsX H66T sample, and no signal accumulated in the T6 UvsX M71F/S72G sample. It was concluded that T6 UvsX S164V/A166S performs well in these assays, however in some later experiments little or no difference to the wild type T6 UvsX was found. It was further concluded that T6 UvsX H66T has poor activity, and T6 UvsX M71F/S72G is inactive.

The sequence of T6 UvsX S164V/A166S is as follows: MSIADLKSRL
IKASTSKMTA ELTTSKFFNE KDVIRTKIPM LNIAISGAID GGMQSGLTIF
AGPSKHFKSN MSLTMVAAYL NKYPDAVCLF YDSEFGITPA YLRSMGVDPE
RVIHTPIQSV EQLKIDMVNQ LEAIERGEKV IVFIDSIGNM ASKKETEDAL
NEKV~~V~~VSDMTR AKSLKSLFRI VTPYFSIKNI PCVAVNHTIE TIEMFSKTVM
TGGTGVMYSA DTVFIIGKRQ IKDGSDLQGY QFVLNVEKSR TVKEKSKFFI
DVKFDGGIDP YSGLLDMALE LGFVVKPKNG WYAREFLDEE TGEMIREEEKS
WRAKDTNCTT FWGPLFKHQP FRDAIKRAYQ LGAIDSNEIV EAEVDELINS
KVEKFKSPES KSKSAADLET DLEQLSDMEE FNE (SEQ ID NO:106).

20 **EXAMPLE 5: RPA using Rb69 Components**

RPA reactions were established using Rb69 components, using primers J1 and K2 under the following conditions: 50mM Tris.acetate pH 7.9, 100 mM Potassium acetate, 14mM Magnesium acetate, 50mM Creatine phosphate (Calbiochem), 3mM ATP (Roche), 200 micromolar dNTPs, 50 ng/μl creatine kinase (Roche), 100 ng/μl UvsX of Rb69, 20-100 ng/μl Rb69 UvsY, 400 ng/μl Rb69 gp32, 30 ng/μl Bsu polymerase, 7% Carbowax 20M, 300nM amplification primers, 1:50,000 dilution from stock of SYBR green (Invitrogen). Reactions were established on ice in a 384-well plate, and then transferred to a BIOTEK Flx-800 fluorescence microplate reader with stage set to 38°C at which time measurements were taken periodically from a bottom-reading probe. Samples contained either no target (control - water) or 2500 copies of *B.subtilis* genomic DNA containing the target sequence. Samples contained varying concentrations of Rb69 UvsY, and the quantities used are indicated in the legend.

As shown in Figure 14, positive signals developed in all samples during the 90 minute incubation, and the time of signal increase was earlier in the samples containing higher quantities of UvsY underlying an ideal requirement for concentrations of Rb69 UvsY of 60ng/μl or over. The control sample was performed under identical conditions to the positive sample containing 60ng/μl of UvsY, but lacking target DNA. This experiment shows that Rb69 components can be employed to configure a sensitive and specific amplification system.

EXAMPLE 6: RPA using Aeh1 Components

RPA reactions were established using Aeh1 components, using primers J1 (120nM) and K2 (480nM) under the following conditions: 50mM Tris acetate pH 7.9, 100 mM Potassium acetate, 14mM Magnesium acetate, 50mM Creatine phosphate (Calbiochem), 3mM ATP (Roche), 200 micromolar dNTPs, 50 ng/μl creatine kinase (Roche), 200 ng/μl Aeh1 UvsX, 80ng/μl Aeh1 UvsY, 500ng/μl Aeh1 gp32, 30 ng/μl Bsu polymerase, 7% PEG compound, 120nM fluorescent probe BsFlc. Exonuclease III was included at 65ng/μl. Reactions were established on ice in a 384-well plate, and then transferred to a BIOTEK Flx-800 fluorescence microplate reader with stage set to 38°C at which time measurements were taken periodically from a bottom-reading probe. Samples contained either water, 10, 100, or 1000 copies of *B.subtilis* genomic DNA containing the target sequence as indicated in the legend shown in Figure 15.

Salt Titration

RPA reactions were also established using Aeh1 components testing salt titration, using primers J1 and K2 under the following conditions: 50mM Tris acetate pH XX, 60 or 80 or 100 or 120 or 140 or 160mM Potassium acetate, 14mM Magnesium acetate, 50mM Creatine phosphate (Calbiochem), 3mM ATP (Roche), 200 micromolar dNTPs, 50 ng/μl creatine kinase (Roche), 150 ng/μl UvsX of Aeh1, 50 ng/μl Aeh1 UvsY, 500ng/μl Aeh1 gp32, 30 ng/μl Bsu polymerase, 7% Carbowax 20M, 300nM amplification primers, 1:50,000 dilution from stock of SYBR green (Invitrogen). Reactions were established on ice in a 384-well plate, and then transferred to a BIOTEK Flx-800 fluorescence microplate reader with stage set to 38°C at which time measurements were taken periodically from a bottom-reading probe. Samples contained 2000 copies of *B.subtilis* genomic DNA containing the target sequence.

As shown in Figure 16, positive signals developed in all samples during the 90 minute incubation. This experiment suggests that Aeh1 components can be employed successfully to amplify DNA over a broad range of salt concentrations.

Aeh1 compared to T4

- 5 RPA reactions were established to compare Aeh1 amplification to the T4 amplification system, using primers orfx45a (100ng/μl) and scii35IV (500ng/μl) under the following conditions: 50mM Tris.acetate pH 7.9, 100 mM Potassium acetate, 14mM Magnesium acetate, 50mM Creatine phosphate (Calbiochem), 3mM ATP (Roche), 200 micromolar dNTPs, 50 ng/μl creatine kinase (Roche), 200 ng/μl Aeh1 UvsX, 80ng/μl Aeh1 UvsY, 500ng/μl Aeh1 gp32, 70 ng/μl Bsu polymerase, 7% PEG Compound (Sigma), 120nM fluorescent probe SATamra2, OR under similar conditions but with the following recombination components: 120ng/μl T4 UvsX, 30ng/μl T4 UvsY and 900 ng/μl T4 gp32. Exonuclease III was included at 65ng/μl. Reactions were established on ice in a 384-well plate, and then transferred to a BIOTEK Flx-800 fluorescence microplate reader with stage set to 38°C at which time measurements were taken periodically from a bottom-reading probe. Samples contained either water, 10 or 1000 copies of MRSA genomic DNA containing the target sequence as indicated in the legend. As shown in Figure 17, no signals were detected with either recombination system when an estimated 10 copies had been provided. Based on later experiments it was believed that the DNA dilutions used for this experiment were compromised and hence that actual copy numbers were significantly lower than those expected. As shown in Figure 17, the Aeh1 recombination system reaches detection threshold later than T4 and achieves a lower total signal strength in this experiment.
- 10
- 15
- 20

Aeh1 UvsX and UvsY can amplify using heterologous gp32

- 25 RPA reactions were established using primers J1 and K2 under the following conditions: 50mM Tris.acetate pH 7.9, 100 mM Potassium acetate, 14mM Magnesium acetate, 50mM Creatine phosphate (Calbiochem), 3mM ATP (Roche), 200 micromolar dNTPs, 50 ng/μl creatine kinase (Roche), 200 ng/μl UvsX of Aeh1, 100 ng/μl Aeh1 UvsY, 300ng/μl Aeh1 gp32 OR 500ng/μl Rb69 gp32 OR 700 ng/μl T4 gp32, 30 ng/μl Bsu polymerase, 7% Carbowax 20M, 300nM amplification primers, 1:50,000 dilution from stock of SYBR green (Invitrogen). Reactions were established on ice in a 384-well plate, and then transferred to a BIOTEK Flx-800 fluorescence microplate reader with stage set to 38°C at
- 30

which time measurements were taken periodically from a bottom-reading probe. Samples contained 2000 copies of *B.subtilis* genomic DNA containing the target sequence.

As shown in Figure 18, signals developed in all samples indicating that DNA amplification had occurred in all cases. The fastest and strongest signals developed when Aeh1 gp32 was employed, then Rb69 gp32, then T4 gp32. One should interpret the relative effectiveness of the gp32 molecules cautiously as they were not employed at the same concentrations.

EXAMPLE 7: RPA using Heterologous Reaction Components

RPA reactions were established using primers Apo300 and ApoB4 which amplify a roughly 300 base pair duplex product from human genomic DNA. The following conditions were employed: 50mM Tris.acetate pH 8.3, 100 mM Potassium acetate, 14mM Magnesium acetate, 50mM Creatine phosphate (Calbiochem), 3mM ATP (Roche), 200 micromolar dNTPs, 50 ng/μl creatine kinase (Roche), 200 ng/μl UvsX of KVP40, Aeh1 or Rb69, 32ng/μl UvsY of KVP40, Aeh1 or T4 as indicated, 600ng/μl Rb69 gp32 or T4 gp32, 30 ng/μl Bsu polymerase, 5% Carbowax 20M, 300nM amplification primers. Reactions were established and left at 37°C for 90 minutes. All samples contained 1000 copies of human genomic DNA containing the target sequence. The precise composition of each reaction with regard to species of gp32, UvsX and UvsY is indicated. Samples were cleaned by passage through a Qiagen PCR clean-up column and electrophoresed on a 2% agarose gel containing ethidium bromide. As shown in Figure 19, amplification had occurred in the sample containing a heterologous mixture of Rb69 gp32 with Aeh1 UvsX and UvsY.

EXAMPLE 8: Engineered Rb69 Constructs

Alterations to our parent clone of Rb69 UvsX in a modified pET21+ vector were engineered. The overall layout of the coding/primary amino acid sequence of RB69 drawing attention to regions of interest is shown at the top of the Figure 20. Changes in the coding sequence were engineered, specifically to alter encoded amino acids in and around the Walker A motif, in and around the DNA-binding loop 2, and at the very C-terminus of the protein. Alterations in and around the Walker motif are as indicated by specific lettering and numeration referring to the position of the amino acid in the Rb69 wild-type protein, what the amino acid is, and to what it is mutated. For example H64S refers to alteration of histidine 64 of the native protein to a serine. Altered sequences in the region of DNA-binding loop 2 are indicated according to a different scheme. In this case most or all of the DNA binding loop

sequences was replaced by the loop from T6 or T4 UvsX. When T6-1 is shown, this refers to replacement of the sequence NHT AMEIGGLYPKE IMG GG (SEQ ID NO:107) with the sequence NHT IETIEMFSKT VMG GG (SEQ ID NO:108), in which the underlined glycine is similar to the Rb69 sequences not the T6 native sequence. When T6 is shown, this refers to replacement of the Rb69 sequence with NHT IETIEMFSKT VMT GG (SEQ ID NO:109), in which the underlined threonine is the native T6 sequence in this position. When T4 is shown, this refers to replacement of the Rb69 sequence with the T4 sequence, that is NHT YETQEMFSKT VMG GG (SEQ ID NO:110). In the case of modifications to the C terminus the symbol 'LSD' indicates alteration of the native sequence of Rb69 at the very C terminus from the encoded amino acid sequence END LDE MEDFDE (SEQ ID NO:111) to the sequence END LDE LSD MEDFDE (SEQ ID NO:112). The symbol 'LDE LDE' or sometimes in the legends '2xLDE' refers to changing the Rb69 C-terminal sequence to END LDE MEDFDE LDE LDE (SEQ ID NO:113). Note that in all cases the very C-terminal sequence is followed by 18 bases encoding 6 histidine residues that are used for protein purification.

Briefly, selected sequences discussed above are listed below.

The Rb69 UvsX H64S sequence is as follows: MSDLKSRLIK ASTSKMTADL
TKSKLFNNRD EVPTRIPMLN IALGGALNAG LQSGLTIFAA PSKSFKTLFG
LTMVAAYMKK YKDAICLFYD SEFGASESYF RSMGVDLDRV VHTPIQSVEQ
20 LKVDMTNQLD AIERGDKVII FIDSIGNTAS KKETEDALNE KVVGDMSRAK
ALKSLFRIVT PYLTIKDIPC VAINHTAMEI GGLYPKEIMG GGTGILYSAN
TVFFISKRQV KEGTELTYD FTLKAEKSRT VKEKSTFPIT VNFDGGIDPF
SGLLEMATEI GFVVKPKAGW YAREFLDEET GEMIREEKSW RAKATDCVEF
WGPLFKHKPF RDAIETKYKL GAISSIKEVD DAVNDLINCK ATTKVPVKTS
25 DAPSAADIEN DLDEMEDEFE (HHHHHH) (SEQ ID NO:114). The six "H" at the end is optional.

The Rb69 UvsX H64S LSD sequence is as follows: MSDLKSRLIK ASTSKMTADL
TKSKLFNNRD EVPTRIPMLN IALGGALNAG LQSGLTIFAA PSKSFKTLFG
LTMVAAYMKK YKDAICLFYD SEFGASESYF RSMGVDLDRV VHTPIQSVEQ
30 LKVDMTNQLD AIERGDKVII FIDSIGNTAS KKETEDALNE KVVGDMSRAK
ALKSLFRIVT PYLTIKDIPC VAINHTAMEI GGLYPKEIMG GGTGILYSAN
TVFFISKRQV KEGTELTYD FTLKAEKSRT VKEKSTFPIT VNFDGGIDPF

SGLLEMATEI GFVVKPKAGW YAREFLDEET GEMIREEKSW RAKATDCVEF
WGPLFKHKPF RDAIETKYKL GAISSIKEVD DAVNDLINCK ATTKVPVKTS
DAPSAADIEN DLDEMEDFDE LSD (HHHHHH) (SEQ ID NO:115). The six "H" at the
end is optional.

- 5 The Rb69 UvsX H64S 2xLDE sequence is as follows: MSDLKSRLIK
ASTSKMTADL TSKSLFNNRD EVPTRIPMLN IALGGALNAG LQSGLTIFAA
PSKSFKTLFG LTMVAAYMKK YKDAICLFYD SEFGASESYF RSMGVDLDRV
VHTPIQSVEQ LKVDMTNQLD AIERGDKVII FIDSIGNTAS KKETEDALNE
KVVGDMSSRAK ALKSLFRIVT PYLTIKDIPC VAINHTAMEI GGLYPKEIMG
10 GGTGILYSAN TVFFISKRQV KEGTELTYD FTLKAESRT VKEKSTFPIT
VNFDGGIDPF SGLLEMATEI GFVVKPKAGW YAREFLDEET GEMIREEKSW
RAKATDCVEF WGPLFKHKPF RDAIETKYKL GAISSIKEVD DAVNDLINCK
ATTKVPVKTS DAPSAADIEN DLDEMEDFDE LSD LDELDE (HHHHHH)(SEQ ID
NO:116). The six "H" at the end is optional.

- 15 The Rb69 UvsX H64S T6/2xLDE sequence is as follows: MSDLKSRLIK
ASTSKMTADL TSKSLFNNRD EVPTRIPMLN IALGGALNAG LQSGLTIFAA
PSKSFKTLFG LTMVAAYMKK YKDAICLFYD SEFGASESYF RSMGVDLDRV
VHTPIQSVEQ LKVDMTNQLD AIERGDKVII FIDSIGNTAS KKETEDALNE
KVVGDMSSRAK ALKSLFRIVT PYLTIKDIPC VAINHTIETI EMFSKTVMTG
20 GTGILYSAN TVFFISKRQV KEGTELTYD FTLKAESRT VKEKSTFPIT
VNFDGGIDPF SGLLEMATEI GFVVKPKAGW YAREFLDEET GEMIREEKSW
RAKATDCVEF WGPLFKHKPF RDAIETKYKL GAISSIKEVD DAVNDLINCK
ATTKVPVKTS DAPSAADIEN DLDEMEDFDE LSD LDELDE (HHHHHH) (SEQ ID
NO:117). The six "H" at the end is optional.

- 25 The Rb69 UvsX H64S T4/2xLDE sequence is as follows: MSDLKSRLIK
ASTSKMTADL TSKSLFNNRD EVPTRIPMLN IALGGALNAG LQSGLTIFAA
PSKSFKTLFG LTMVAAYMKK YKDAICLFYD SEFGASESYF RSMGVDLDRV
VHTPIQSVEQ LKVDMTNQLD AIERGDKVII FIDSIGNTAS KKETEDALNE
KVVGDMSSRAK ALKSLFRIVT PYLTIKDIPC VAINHTYETQ EMFSKTVMGG
30 GTGILYSAN TVFFISKRQV KEGTELTYD FTLKAESRT VKEKSTFPIT
VNFDGGIDPF SGLLEMATEI GFVVKPKAGW YAREFLDEET GEMIREEKSW
RAKATDCVEF WGPLFKHKPF RDAIETKYKL GAISSIKEVD DAVNDLINCK

ATTKVPVKTS DAPSAADIEN DLDEMEDFDE LSD **LDELDE** (HHHHHH)(SEQ ID NO:118). The six "H" at the end is optional.

The Rb69 UvsX H64S T67S L68N T4/2xLDE sequence is as follows:

MSDLKSRLIK ASTSKMTADL TSKSLFNNRD EVPTRIPMLN IALGGALNAG
 5 LQSGLTIFAA PSK**SFKSN**FG LTMVAAYMKK YKDAICLFYD SEFGASESYF
 RSMGVDLDRV VHTPIQSVEQ LKVDMTNQLD AIERGDKVII FIDSIGNTAS
 KKETEDALNE KVVGDMSRAK ALKSLFRIVT PYLTIKDIPC VAIN**NHTYETO**
EMFSKTVMGG GTGILYSAN TVFFISKRQV KEGTELTGYD FTLKAESRT
 VKEKSTFPIT VNFDGGIDPF SGLLEMATEI GFVVKPKAGW YAREFLDEET
 10 GEMIREEKSW RAKATDCVEF WGPLFKHKPF RDAIETKYKL GAISSIKEVD
 DAVNDLINCK ATTKVPVKTS DAPSAADIEN DLDEMEDFDE LSD **LDELDE**
 (HHHHHH) (SEQ ID NO:119). The six "H" at the end is optional.

Additional alterations to the parent clone of Rb69 UvsX in a modified pET21+ vector were generated. The overall layout of the coding/primary amino acid sequence of Rb69
 15 drawing attention to additional regions of interest is shown at the top of the Figure 21. Changes in the coding sequence were engineered, specifically in and around the DNA-binding loop 2. The entire DNA-binding loop2 sequence was replaced with the equivalent sequences from phage 133, phage Aeh1, phage KVP40, a representative (hybrid) cyanophage sequence, or the loop from *E.coli* RecA. A loop which was part Aeh1 and part Rb16 was also
 20 tested. The precise amino acid substitutions are indicated in Figure 21. A summary remark regarding the behaviour/activity of the protein produced from these clones during expression/purification or testing in RPA is given on the left of Figures 20 and 21.

The Rb69 UvsX sequence is as follows: MSDLKSRLIK ASTSKMTADL
 TSKSLFNNRD EVPTRIPMLN IALGGALNAG LQSGLTIFAA PSKHFKTLFG
 25 LTMVAAYMKK YKDAICLFYD SEFGASESYF RSMGVDLDRV VHTPIQSVEQ
 LKVDMTNQLD AIERGDKVII FIDSIGNTAS KKETEDALNE KVVGDMSRAK
 ALKSLFRIVT PYLTIKDIPC VAIN**HTAMEI** **GGLYPKEIMG** **GGT**GILYSAN
 TVFFISKRQV KEGTELTGYD FTLKAESRT VKEKSTFPIT VNFDGGIDPF
 SGLLEMATEI GFVVKPKAGW YAREFLDEET GEMIREEKSW RAKATDCVEF
 30 WGPLFKHKPF RDAIETKYKL GAISSIKEVD DAVNDLINCK ATTKVPVKTS
 DAPSAADIEN DLDEMEDFDE (SEQ ID NO:120)

The Rb69 Loop133 UvsX sequence is as follows: MSDLKSRLIK ASTSKMTADL
 TSKLFFNNRD EVPTRIPMLN IALGGALNAG LQSGLTIFAA PSKHFCTLFG
 LTMVAAYMKK YKDAICLFYD SEFGASESYF RSMGVDLDRV VHTPIQSVEQ
 LKVDMTNQLD AIERGDKVII FIDSIGNTAS KKETEDALNE KVVGDMSRAK
 5 ALKSLFRIVT PYLTIKDIPC VAI NHT LQTFLEMFSEVMT GGTGILYSAN
 TVFFISKRVQ KEGTELTGYD FTLKAEKSRT VKEKSTFPIT VNFDGGIDPF
 SGLLEMATEI GFVVKPKAGW YAREFLDEET GEMIREEKSW RAKATDCVEF
 WGPLFKHKPF RDAIETKYKL GAISSIKEVD DAVNDLINCK ATTKVPVKTS
 DAPSAADIEN DLDEMEDFDE (SEQ ID NO:121)

10 The Rb69 LoopKVP40 UvsX sequence is as follows: MSDLKSRLIK
 ASTSKMTADL TSKLFFNNRD EVPTRIPMLN IALGGALNAG LQSGLTIFAA
 PSKHFCTLFG LTMVAAYMKK YKDAICLFYD SEFGASESYF RSMGVDLDRV
 VHTPIQSVEQ LKVDMTNQLD AIERGDKVII FIDSIGNTAS KKETEDALNE
 KVVGDMSRAK ALKSLFRIVT PYLTIKDIPC VAI NHT YQTQEIYSKTVMS
 15 GGTGILYSAN TVFFISKRVQ KEGTELTGYD FTLKAEKSRT VKEKSTFPIT
 VNFDGGIDPF SGLLEMATEI GFVVKPKAGW YAREFLDEET GEMIREEKSW
 RAKATDCVEF WGPLFKHKPF RDAIETKYKL GAISSIKEVD DAVNDLINCK
 ATTKVPVKTS DAPSAADIEN DLDEMEDFDE (SEQ ID NO:122)

Activity of Rb69 H64S

20 A kinetic study of the activity of mutant Rb69 H64S protein compared to wild type
 Rb69, or T4 UvsX, was made. A fluorescent probe based monitoring approach was taken.
 General conditions were as for the experiment shown in Figure 13 with the exception of the
 type and concentrations of recombination components, and that PEG compound was
 employed at 7% w/v. Other changes are as follows: 120 ng/μl T4 UvsX, 900 ng/μl T4 gp32,
 25 50ng/μl T4 UvsY, OR 100ng/μl Rb69 or Rb69 H64S UvsX, 400ng/μl Rb69 gp32, 80ng/μl
 Rb69 UvsY. Target DNA was present at 100 copies total. As shown in Figure 22, the Rb69
 H64S protein works well according to this assay (although this experiment does not address
 the nature of the DNA generated during amplification) and seems to outperform the kinetics
 of the wild-type protein. In the next experiment performed the rate under apparently identical
 30 conditions (400 ng/μl Rb69 gp32) the outcome was slightly different. This is most likely due
 to slight pipetting errors in the latter experiment.

Rb69 H64S – relative resistance to gp32 up-titration

A kinetic study of the activity of mutant Rb69 H64S protein compared to wild type Rb69 was made in which the quantity of Rb69 gp32 was varied somewhat. A fluorescent probe based monitoring approach was taken. General conditions were as for the experiment shown in Figure 22 with the exception of a variable concentration of gp32 protein, and that PEG compound was employed at 6% w/v. Conditions were: 100ng/μl Rb69 or Rb69 H64S UvsX, Rb69 gp32 concentration as indicated, 80ng/μl Rb69 UvsY. Target DNA was present at 100 copies total. As shown in Figure 23, up-titration of gp32 had less impact on kinetics of Rb69 H64S compared to Rb69 protein. It was concluded that RbH64S is somewhat more resistant to competition by gp32.

10 *Activity of Rb69 H64S compared to wild type Rb69*

A kinetic study of the activity of mutant Rb69 H64S protein compared to wild type Rb69 was made. A fluorescent probe based monitoring approach was taken. General conditions were as for the experiment shown in Figure 22 with the exception of the type and concentrations of recombination components, and that PEG compound was used at 6% w/v. Other conditions are as follows: 100ng/μl Rb69 or Rb69 H64S UvsX, 400ng/μl Rb69 gp32, 80ng/μl Rb69 UvsY. Target DNA was present at 0 copies, 100 copies, or 1000 copies total as indicated. As shown in Figure 24, the Rb69 H64S protein works well according to this assay and outperforms the behaviour of the wild-type protein.

Activity of Rb69 UvsX H64S at 300-500 ng/μl gp32

20 A kinetic study of the activity of mutant Rb69 H64S protein was made under conditions of 300, 400, or 500 ng/μl of Rb69 gp32 protein. A fluorescent probe based monitoring approach was taken. General conditions were as for the experiment shown in Figure 22 but gp32 concentrations were varied and PEG compound was used at 6% w/v. Protein concentrations were thus as follows: 100ng/μl Rb69 H64S UvsX, 300-500ng/μl Rb69 gp32, 80ng/μl Rb69 UvsY. Target DNA was present at 0 (water control) or 100 copies total as indicated. As shown in Figure 25, the Rb69 H64S protein works well according to this assay with little difference in kinetic behaviour over the tested range of Rb69 gp32 protein.

Titration of Rb69 UvsX H64S

30 A kinetic study of the activity of mutant Rb69 H64S UvsX protein was made under varying concentrations of UvsX protein. A fluorescent probe based monitoring approach was taken. General conditions were as for the experiment shown in Figure 22 but the concentration of Rb69 H64S UvsX was varied and PEG compound was used at 6% w/v.

Protein concentrations were thus as follows: 100, 150 or 200 ng/μl Rb69 H64S UvsX, 500ng/μl Rb69 gp32, 80ng/μl Rb69 UvsY. Target DNA was present at 0 (water control) or 100 copies total as indicated. As shown in Figure 26, the Rb69 H64S protein works well according to this assay providing that the UvsX concentration does not significantly exceed 100ng/μl.

Another kinetic study of the activity of mutant Rb69 H64S protein was performed under varying concentrations of UvsX protein using a fluorescent probe based monitoring approach. General conditions were as for the experiment shown in Figure 22 but the concentration of Rb69 H64S UvsX was varied and PEG compound was employed at 6%w/v. Protein concentrations were thus as follows: 60, 80 or 100 ng/μl Rb69 H64S UvsX, 500ng/μl Rb69 gp32, 80ng/μl Rb69 UvsY. Target DNA was present at 0 (water control) or 100 copies total as indicated. As shown in Figure 27, the Rb69 H64S protein works well according to this assay regardless of whether the protein was in the range 60-100ng/μl.

Effectiveness of Rb69 gp32 in reactions with T4 UvsX and UvsY

A kinetic study investigating the utility of Rb69 gp32 when combined with T4 UvsX and UvsY was performed. RPA reactions were established using primers J1 (120ng/μl) and K2 (480 ng/μl) under the following conditions: 50mM Tris.acetate pH 7.9, 100 mM Potassium acetate, 14mM Magnesium acetate, 50mM Creatine phosphate (Calbiochem), 3mM ATP (Roche), 200 micromolar dNTPs, 50 ng/μl creatine kinase (Roche), 120 ng/μl T4 UvsX, 30ng/μl T4 UvsY, 900ng/μl T4 gp32 OR 500 ng/μl Rb69 gp32 OR 1000 ng/μl , 30 ng/μl Bsu polymerase, 6% PEG 35,000, 120nM fluorescent probe BsFlc. Exonuclease III was included at 65ng/μl. Reactions were established on ice in a 384-well plate, and then transferred to a BIOTEK Flx-800 fluorescence microplate reader with stage set to 38°C at which time measurements were taken periodically from a bottom-reading probe. Samples contained either water or 100 copies of *B.subtilis* genomic DNA containing the target sequence as indicated in the legend. As shown in Figure 28, all template positive samples worked effectively and there appeared to be little difference between using T4 and Rb69 gp32 protein.

T4 outperforms Rb69 UvsX/UvsY system when Rb69 gp32 is used in both cases

A kinetic study investigating the utility of Rb69 gp32 when combined with T4 UvsX and UvsY, or when combined with Rb69 UvsX and UvsY. RPA reactions were established using primers J1 (120 nM) and K2 (480 nM) under the following conditions: 50mM

Tris.acetate pH 7.9, 100 mM Potassium acetate, 14mM Magnesium acetate, 50mM Creatine phosphate (Calbiochem), 3mM ATP (Roche), 200 micromolar dNTPs, 50 ng/μl creatine kinase (Roche), 120 ng/μl T4 UvsX, 30 ng/μl T4 UvsY, 1000 ng/μl Rb69 gp32, 30 ng/μl Bsu polymerase, 6% PEG 35,000, 300nM amplification primers, 120nM fluorescent probe

5 BsFlc. Exonuclease III was included at 65ng/μl. Alternatively similar conditions were employed but the recombinase was 100 ng/μl Rb69 UvsX and the loading protein was 80 ng/μl Rb69 UvsY protein. Reactions were established on ice in a 384-well plate, and then transferred to a BIOTEK Flx-800 fluorescence microplate reader with stage set to 38°C at which time measurements were taken periodically from a bottom-reading probe. Samples

10 contained either water or 100 copies of *B.subtilis* genomic DNA containing the target sequence as indicated in the legend. As shown in Figure 29, all template positive samples developed positive signals, however the system established with T4 UvsX and UvsY develop much earlier and stronger signals. It was concluded that when the Rb69 gp32 concentration is raised to 1000 ng/μl Rb69 little inhibition of amplification occurs when the T4 components

15 are used, but when Rb69 UvsX and UvsY are used there is significant inhibition (see effects of Rb69 gp32 overtitration with Rb69 UvsX and UvsY in Figure 23).

Poor activity of Rb69 UvsX H64T protein

An RB69 UvsX-encoding clone was generated in which histidine 64 was altered to a threonine. This mutation was analogous to the Rb69 UvsX H64S protein assessed earlier, and

20 was designed to test whether a threonine residue would be as effective as a serine residue at improving RPA behaviour. General reaction conditions were the same as described for the experiment in Figure 29 with the following exceptions: UvsX was either Rb69 wild type UvsX at 100 ng/μl or Rb69 UvsX H64T at 100 ng/μl, Rb69 UvsY at 80 ng/μl, and 500 ng/μl Rb69 gp32. DNA target was present either at 0 or 100 copies. As shown in Figure 30,

25 reactions performed using Rb69 UvsX H64T barely developed signal and it was deduced that this amino acid substitution is not effective in contrast to when a serine is substituted at this position.

ATP titration using Rb69 UvsX

The effects of different ATP concentrations on the amplification kinetics when using

30 Rb69 UvsX protein were investigated. Reaction conditions were as in Figure 30 but only wild-type Rb69 gp32, UvsX, and UvsY were used. The final concentration of ATP was adjusted to either 1mM, 2mM, or 3mM. Target was present at either 0 or 100 copies as

indicated. As shown in Figure 31, amplification occurred in all cases that target DNA was present, but the strongest signals develop when 3 mM ATP is used.

Suppressing effect of T4 gp32 on Rb69 UvsX and UvsY

The effects using T4 gp32 protein with Rb69 UvsX and UvsY proteins were
5 investigated. Conditions were the same as those described in Figure 29 with the following modifications. Rb69 UvsX was used at 100 ng/μl, Rb69 UvsY was used at 80 ng/μl, and gp32 was either Rb69 gp32 at 500 ng/μl OR T4 gp32 at 500 ng/μl OR T4 gp32 at 1000 ng/μl. As shown in Figure 32, signals only develop when Rb69 gp32 is used, and not when T4 gp32 is employed contrasting with the full compatibility of Rb69 gp32 when used with T4
10 heterologous components.

Consequences of modification to the C terminus of Rb69 UvsX

A kinetic analysis of amplification reactions configured with Rb69 UvsX H64S, with Rb69 UvsX H64S LSD, and with Rb69 UvsX H64S 2xLDE was performed. General reaction conditions were as described in Figure 29, except that different UvsX proteins were used in
15 all cases at 100 ng/μl. Rb69 UvsY was used at 80 ng/μl. Rb69 gp32 was used at 500 ng/μl. DNA target was present at either 0 or 1000 copies. As shown in Figure 33, strong signals develop in all target-containing samples and show similar kinetics. A very slight tendency for the proteins with more acidic C-termini (LSD and 2xLDE clones) to initiate signal very slightly later and to generate slightly stronger signals in total is seen.

20 A similar experiment to that described in Figure 33 was performed. However in this case DNA target was present at either 0 or 100 copies. As shown in Figure 34, strong signals develop in all target-containing samples and show, once again, fairly similar kinetics. In this case, a slightly stronger tendency for the proteins with more acidic C-termini (LSD and 2xLDE clones) to initiate signal slightly later and to generate stronger signals was observed.

25 *Titration of PEG when using Rb69 UvsX H64S/2xLDE*

Similar conditions were employed as in the experiment described in Figure 33. However in this case only Rb69 UvsX H64S 2xLDE was used and at a concentration of 100ng/μl, Rb69 UvsY was used at 80ng/μl, and Rb69 gp32 was used at 500 ng/μl. DNA target was present at either 0 or 200 copies per reaction as indicated. The concentration of
30 polyethylene glycol (M.W. 35,000 Fluka) was tested at 5%, 6%, and 7%. As shown in Figure 35, the best signals were obtained when polyethylene glycol M.W. 35,000 was used at 5% w/v.

EXAMPLE 9: Engineered UvsY Constructs

A schematic representation is shown of the peptide sequence predicted to be encoded by the T4 UvsY and Rb69 UvsY genes is shown in Figure 36. Residues that are substituted between these 2 proteins are indicated, all other residues are identical. Two chimeric clones
 5 which were used to express chimeric proteins were generated. Each chimera consisted of the N-terminal half of one UvsY molecule fused to the C-terminal half of the other. These are termed UvsY hybrid 1 and UvsY hybrid 2.

Activity of UvsY hybrids with T4 UvsX and T4 gp32

An experiment was performed to address how well the T4, Rb69, and hybrid UvsY
 10 proteins described in Figure 36 would function when combined with T4 UvsX and T4 gp32. Standard conditions as described for the experiment in Figure 29 were used but with the following modifications. T4 UvsX was employed at a concentration of 120 ng/μl, T4 gp32 was employed at 900 ng/μl, and the UvsY proteins tested were used at 80ng/μl. DNA target was present at either 0 or 1000 copies in each reaction. PEG 35,000 (Fluka) was employed at
 15 5% w/v. As shown in Figure 37, all of the different forms of UvsY behaved excellently in this assay indicating that when T4 UvsX and T4 gp32 are employed there is little or no preference visible for T4 vs Rb69 UvsY, nor any significant distinction from the hybrid molecules.

Activity of UvsY hybrids with Rb69 UvsX and Rb69 gp32.

20 An experiment was performed to address how well the T4, Rb69, and hybrid UvsY proteins described in Figure 36 would function when combined with Rb69 UvsX and Rb69 gp32. Standard conditions as described for the experiment in Figure 37 were used but with the following modifications. Rb69 UvsX H64S 2xLDE was employed at a concentration of 100 ng/μl, Rb69 gp32 was employed at 500 ng/μl, and the UvsY proteins tested were used at
 25 80ng/μl. DNA target was present at either 0 or 1000 copies in each reaction. As shown in Figure 38, all the forms of UvsY functioned in this assay, however there were strong differences in response time and signal strength. This indicates that when Rb69 UvsX and Rb69 gp32 are employed there is a clear preference for Rb69 UvsY.

The sequence of UvsY hybrid 1 is as follows: HHHHHHMRLEDLQEEL
 30 KKDV FIDSTK LQYEAANNVM LYSKWL NKHS SIKKEMLR IE AQKKVAL KAR
 LDYYSGRGDG DEFSMDRYEK SEMKTVLAAD KDVLKIETTL QYWGILLEFC

SGALDAVKSR SFALKHIQDM REFEAGQ (SEQ ID NO:123). The N terminus six histidines are optional.

The sequence of UvsY hybrid 2 is as follows: HHHHHHMKLEDLQEEL
DADLAIDTTK LQYETANNVK LYSKWLRKHS FIRKEMLRIE TQKKT ALKAR
5 LDYYSGRGDG DEFSMDRYEK SEMKTVLSAD KDVLKVDTSI QYWGILLDFC
SGALDAIKSR GFAIKHIQDM RAFEAGK (SEQ ID NO:124). The N terminus six histidines are optional.

EXAMPLE 10: Additional Analysis of Rb69 Engineered Constructs and Chimeras

No activity for Rb69 UvsX H64S/T6-1/2xLDE

10 The activity of Rb69 UvsX H64S/T6-1 2xLDE in comparison to the robust activity of Rb69 UvsX H64S/2xLDE was investigated. Reactions were established according to standard conditions described in Figure 29 with the following modifications. Rb69 UvsX H64S/2xLDE protein and Rb69 UvsX H64S/T6-1/2xLDE protein were used at 100 ng/μl, Rb69 gp32 was used at 600 ng/μl, and Rb69 UvsY was employed at 80ng/μl. DNA target
15 was present at either 0 or 1000 copies per reaction. As shown in Figure 39, robust activity was exhibited by the Rb69 UvsX H64S/2xLDE protein, but no activity was detected with Rb69 UvsX H64S/T6-1/2xLDE protein. Apparently recoding the DNA-binding loop 2 sequence in this case resulted in a non-functional protein.

Titration of Rb69 gp32 in the presence of Rb69 UvsX H64S/2xLDE

20 The effects of titrating Rb69 gp32 protein on amplification kinetics when employing the Rb69 UvsX/H64S 2xLDE protein were investigated. Reactions were established according to standard conditions described in Figure 29 with the following modifications. PEG 35,000 (Fluka) was used at 5% w/v. Rb69 UvsX H64S/2xLDE protein was used at 100 ng/μl, Rb69 gp32 was used at 400, 700, or 1000 ng/μl, and Rb69 UvsY was employed at
25 80ng/μl. DNA target was present at either 0 or 100 copies per reaction. As shown in Figure 40, increasing quantities of Rb69 gp32 lead to a delay in onset of signal detection.

No activity for Rb69 UvsX H64S/F69M/G70S/T6-1/2xLDE

The effects of using Rb69 UvsX H64S/F69M/G70S/T6-1/2xLDE protein in amplification reactions were investigated. This clone was similar to that tested earlier
30 containing most of the T6 UvsX DNA-binding loop 2, but also contained 2 additional T6-like residues near to the Walker A motif. Reactions were established according to standard conditions described in Figure 40 with the following modifications. Rb69 UvsX

H64S/2xLDE protein or Rb69 UvsX H64S F69M/G70S/T6-1/2xLDE were used at 100 ng/μl, Rb69 gp32 was used at 500 ng/μl, and Rb69 UvsY was employed at 80ng/μl. DNA target was present at either 0 or 1000 copies per reaction. As shown in Figure 41, no activity is detected for the Rb69 UvsX H64S F69M/G70S/T6-1/2xLDE protein.

5 *Strong activity of Rb69 H64S T67S/L68N/T4/2xLDE and Rb69 H64S/T4/2xLDE*

The effects of using Rb69 H64S T67S/L68N/T4/2xLDE and Rb69 H64S/T4/2xLDE protein in amplification reactions were investigated. These proteins were analogous to those tested earlier containing T6 UvsX DNA-binding loop 2 and/or additionally containing T6-like residues near to the Walker A motif, except that in this case the DNA-binding loop2
10 sequences and Walker A sequences were derived from T4 UvsX (see clone schematic chart). Reactions were established according to standard conditions described in Figure 40 with the following modifications. Rb69 UvsX protein or Rb69 UvsX H64S/2xLDE or Rb69 UvsX H64S/T67S/L68N/T4/2xLDE were used at 100 ng/μl, Rb69 gp32 was used at 500 ng/μl, and Rb69 UvsY was employed at 80ng/μl. DNA target was present at either 0 or 100 copies per
15 reaction. As shown in Figure 42, excellent activity was detected for all UvsX proteins tested indicating that the T4 DNA-binding loop and associated Walker A residues may be substituted successfully into the Rb69 UvsX protein.

Rb69 UvsX H64S/T67S/L68N/ T4/2xLDE protein is relatively resistant to up-titration of Rb69 gp32

20 The inhibitory effect of overtitation of Rb69 gp32 on reaction kinetics comparing wild-type Rb69 UvsX and Rb69 UvsX H64S/T67S/L68N/T4/2xLDE was investigated. Reactions were established according to standard conditions described in Figure 40 with the following modifications. Rb69 UvsX protein or Rb69 UvsX H64S/T67S/L68/T4/2xLDE were used at 100 ng/μl, Rb69 gp32 was used at either 400 or 800 ng/μl, and Rb69 UvsY was
25 employed at 80ng/μl. DNA target was present at either 0 or 100 copies per reaction. As shown in Figure 43, the slowing in time to detection experienced for Rb69 UvsX H64S/T67S/L68N/T4/2xLDE compared to wild-type Rb69 UvsX when increasing the gp32 concentration was only about half as much. It was concluded that the substituted protein is less sensitive to gp32 concentration.

30 *Rb69 UvsX H64S/T67S/L68N/T4/2xLDE protein can function with T4 gp32*

Whether or not the inhibitory effect of T4 gp32 on reactions configured with Rb69 UvsX and UvsY could be overcome by the use of Rb69 UvsX H64S/T67S/L68N/T4/2xLDE

was investigated. Reactions were established according to standard conditions described in Figure 40 with the following modifications. T4 UvsX protein or Rb69 UvsX or Rb69 UvsX H64S/T67S/L68N/T4/2xLDE were used at 120 ng/μl or 100 ng/μl or 100 ng/μl respectively, T4 gp32 was used at 700 ng/μl, and T4 or Rb69 UvsY was employed at 30ng/μl or 80ng/μl respectively. T4 UvsX was combined with T4 UvsY, and the Rb69 UvsX proteins were combined with Rb69 UvsY. DNA target was present at either 0 or 100 copies per reaction. As shown in Figure 44, Rb69 UvsX H64S/T67S/L68N/T4/2xLDE functioned almost as well as the T4 components, while wild-type Rb69 UvsX was inactive when T4 gp32 was used. It was concluded that the substituted Rb69 protein has developed very good tolerance to T4 gp32.

Rb69 UvsX chimeras containing DNA-binding loops from phage 133 work weakly, while cyanophage and Aeh1 loops are non-functional

The activity of Rb69 UvsX proteins in which the DNA-binding loop2 had been replaced with sequences found in other diverse UvsX-like molecules was investigated. Reactions were established according to standard conditions described in Figure 40 with the following modifications. Rb69 UvsX protein or Rb69 UvsX loop 133 or Rb69 loop Cyano or Rb69 loop Aeh1 were used at 100 ng/μl, Rb69 gp32 was used at either 500 ng/μl, and Rb69 UvsY was employed at 80ng/μl. As shown in Figure 45, no activity was detected for the proteins containing cyanophage or Aeh1 loops, while the protein containing the Phage 133 loop showed very weak activity.

Rb69 UvsX H64S/T6/2xLDE is active unlike the equivalent lacking the final G to T substitution of the DNA-binding loop2

The activity of Rb69 UvsX H64S/T6/2xLDE was tested, that is a protein in which the final residue that differs between T4 and T6 has been altered to the T6 equivalent unlike the case with Rb69 UvsX H64S T6-1 2xLDE. Also tested was a protein in which the DNA-binding loop2 had been replaced with a hybrid of the Aeh1 loop and the Rb16 loop (possessing the unusual alanine at the beginning of the Aeh1 loop instead of the cysteine found in Rb16) Reactions were established according to standard conditions described in Figure 40 with the following modifications. Rb69 UvsX protein or Rb69 UvsX H64S/T6/2xLDE or Rb69 loop (hybrid Aeh1/Rb16) were used at 100 ng/μl, Rb69 gp32 was used at either 500 ng/μl, and Rb69 UvsY was employed at 80ng/μl. As shown in Figure 46, no activity was detected for the proteins containing the Aeh1/Rb16 hybrid loop, however the protein containing the repaired T6 loop showed excellent activity. It was concluded that a

complete replacement of the T6-like DNA-binding loop 2 results in activity, but hybrids of the similar T4 and T6 loops are not active indicating that substitutions between T4 and T6 are not silent and must be exchanged in groups.

EXAMPLE 11: Manganese ions are able to support RPA reactions

5 RPA reactions were established under the following conditions: 50mM Tris.acetate pH8.3, 100mM Potassium acetate, 200µM dNTPs, 3mM ATP, 50mM phosphocreatine, 120ng/µl T4 UvsX, 30ng/µl T4 UvsY, 900 ng/µl T4 gp32, 5% PEG 35,000, 30ng/µl Bsu polymerase, 1000 copies *B.subtilis* genomic DNA. Divalent manganese cations were supplied individually to each reaction to give final concentrations of 0.1mM, 0.5mM, 1mM, 2mM, 10 3mM. Alternatively as a control 16mM Magnesium was employed. Reactions were incubated at 37°C for 90 minutes, purified on PCR clean-up columns (SIGMA) and then separated on a 2% agarose gel before visualization with ethidium bromide. As shown in Figure 47, manganese ions efficiently supported RPA in the concentration range of 0.5 to 3mM manganese. Significantly higher concentrations (from about 4-5 mM Manganese – not shown 15 here) started to inhibit reactions behaviour which lead to progressively less product until at 10mM manganese no product was detected with these primers after 90 minutes. Some carry-over of magnesium ions from buffers is anticipated, perhaps accounting for roughly 0.5mM magnesium ions total per reaction.

20 **EXAMPLE 12: *Staphylococcus aureus* polymerase I large fragment functions well in RPA reactions**

RPA reactions were configured using alternative polymerases capable of strand displacement synthesis, including bacterial polymerase I repair enzymes which bear homology to the Pol I class of *E.coli*, *Bacillus subtilis*, and *Staphylococcus aureus*. In this experiment, either the *Bacillus subtilis* PolI large fragment described elsewhere and herein, or 25 with the equivalent large fragment from *S. aureus*, generated in-house were used in RPA reactions. Reactions were configured under standard conditions, namely: 300nM primer J1, 300nM primer K2, 50mM Tris.acetate pH 7.9, 100mM Potassium acetate, 200µM dNTPs, 3mM ATP, 50mM phosphocreatine, 120ng/µl T4 UvsX, 30ng/µl T4 UvsY, 900 ng/µl T4 gp32, 5% PEG compound (SIGMA), 70ng/µl Bsu polymerase OR 70ng/µl *S. aureus* (Sau) 30 polymerase, and 0, 100, 1000 or 10,000 copies *B.subtilis* genomic DNA. Reactions were monitored by the inclusion of 1:50,000 dilution of SYBR green (Invitrogen). As shown in Figure 48, iboth cases robust amplification occurred. If anything the temporal separation

between water and target-containing samples was larger when *S.aureus* polymerase was employed. This could indicate that this polymerase displays slightly improved characteristics for sensitive RPA reactions.

EXAMPLE 13: Use of Heparin in RPA Reactions

5 *Heparin slows the development of signals in zero-target controls*

RPA reactions were configured using the J1 and K2 primers used elsewhere in this disclosure but deliberately omitted target DNA. Reactions were configured under standard conditions, namely: 300nM primer J1, 300nM primer K2, 50mM Tris.acetate pH 7.9, 100mM Potassium acetate, 200μM dNTPs, 3mM ATP, 50mM phosphocreatine, 120ng/μl T4 UvsX,
 10 30ng/μl T4 UvsY, 900 ng/μl T4 gp32, 5% PEG compound (SIGMA), 30ng/μl Bsu polymerase. Reactions were monitored by the inclusion of 1:50,000 dilution of SYBR green (Invitrogen). Heparin was either not included in the reaction, or present at 20ng/μl. As shown in Figure 49, after some time background signals develop in all reactions, however this occurs later for those samples containing heparin suggesting it slows noise development.

15 *Heparin improves signal:noise ratios in RPA reactions*

A kinetic study was made to investigate the effects of heparin on the sensitivity and kinetics of amplification reactions monitored via a probe-based approach. RPA reactions were established using primers J1 (120ng/μl) and K2 (480ng/μl) under the following conditions: 50mM Tris.acetate pH 7.9, 100 mM Potassium acetate, 14mM Magnesium
 20 acetate, 50mM Creatine phosphate (Calbiochem), 3mM ATP (Roche), 200 micromolar dNTPs, 50 ng/μl creatine kinase (Roche), 120 ng/μl T4 UvsX, 30ng/μl T4 UvsY, 1000 ng/μl Rb69 gp32, 30 ng/μl Bsu polymerase, 5% PEG compound, 120nM fluorescent probe BsFlc. Exonuclease III was included at 65ng/μl. Heparin was either absent or present at 20ng/μl as indicated. Reactions were established on ice in a 384-well plate, and then transferred to a
 25 BIOTEK Flx-800 fluorescence microplate reader with stage set to 38°C at which time measurements were taken periodically from a bottom-reading probe. Samples contained either water, 10, 100, 1000 or 10,000 copies of *B.subtilis* genomic DNA containing the target sequence as indicated in the legend. As shown in Figure 50, all template positive samples developed positive signals, however the system established with heparin showed
 30 improvement in the consistency of signal development at 10 copies. It was concluded that the heparin inclusion slowed noise development which led to less of a breakdown of simultaneity of signal detection at low copy numbers.

EXAMPLE 14: 3'-blocked primers and *E.coli* exonuclease III in RPA reactions.

Strong evidence was discovered which suggests that primers which were 3'-blocked with groups such as biotin, via a carbon-oxygen-carbon linkage at least, may be successfully employed as amplification primers if *E.coli* exonuclease III is included in the reaction. This experiment provides an example of this phenomenon. In this experiment RPA reactions were performed by amplifying a fragment from the *Bacillus subtilis* genome using the primers J1 and K2 used widely in this document. The use of a primer designated K2-epsilon which had been designed for other purposes. This primer has the same sequence as the K2 primer, but differs in its possession of a 3'-blocking biotin group which is attached via a linker and described as biotin-TEG (see suppliers website <http://uk.eurogentec.com>). This constitutes a biotin attached via a linker which is joined to the 3' sugar via an oxygen atom. The K2-epsilon primer also contains a deoxyuracil residue replacing a deoxythymidine residue within the body of the sequence, however this is considered of no relevance to this experiment. Reactions contained the J1 primer paired with the K2 primer OR the K2-epsilon 'blocked' primer, and either exonuclease III or *E.coli* Nfo protein. RPA reactions were established using primers J1 (120ng/μl) and K2 or K2 epsilon (480ng/μl) under the following conditions: 50mM Tris acetate pH 7.9, 100 mM Potassium acetate, 14mM Magnesium acetate, 50mM Creatine phosphate (Calbiochem), 3mM ATP (Roche), 200 micromolar dNTPs, 50 ng/μl creatine kinase (Roche), 120 ng/μl T4 UvsX, 30ng/μl T4 UvsY, 1000 ng/μl T4 gp32, 30 ng/μl Bsu polymerase, 5% PEG compound, 120nM fluorescent probe BsFlc. Exonuclease III was included at 65ng/μl or endonuclease IV (Nfo) was included at 200ng/μl. Despite the blocked nature of the K2 epsilon primer, when exonuclease III is used as the agent to process the probe to generate fluorescence, there is no difference in amplification kinetics between samples employing K2 and those employing K2-epsilon, as shown in Figure 51. This suggests that exonuclease III rapidly processes non-extendable hybrids of template bound to K2-epsilon into extendable forms, presumably either by exonuclease activity or via the 3'-diesterase or phosphatase types of activity that have been attributed to this enzyme and Nfo (also known as endonuclease IV). In contrast, when Nfo was employed in place of Exo III, there was a general delay in amplification but this was much more marked for the J1 paired with K2-epsilon reaction. It was concluded that the 'activation' process works poorly when Nfo is employed, but very rapidly when exoIII is employed.

EXAMPLE 15: UvsY-Free DNA amplification

A series of experiments were performed to investigate the effects on DNA amplification by removing UvsY from the RPA reaction.

UvsY-Free DNA amplification using T6 H66S

In this experiment RPA was performed under the following conditions: 100mM Potassium acetate, 50mM Tris.acetate pH 8.3, 14mM Magnesium acetate, 5mM dTT, 200mM dNTPs, 50mM Creatine phosphate (Calbiochem), 2.5mM ATP (Roche), 50ng/μl Creatine Kinase (Roche), 300nM amplification primers, 5% PEG 35,000, 43ng/μl S.au Polymerase, 600ng/μl Rb69 gp32, 120ng/μl T6 H66S UvsX and 79ng/μl Rb69 UvsY, where appropriate. Reactions were carried out using 1000 copies MS2 DNA template with primer MS2 downRT2 and primer MS2 up4, up5, up6 or up7, and in the presence or absence of Rb69 UvsY. Reactions were established on ice and then transferred to 37°C for 1 hour. Following amplification, the products were purified using the GenElute PCR cleanup kit (Sigma) and visualised using gel electrophoresis. Discovered unexpectedly was that T6 H66S recombinase could effectively amplify DNA in RPA reactions in the absence of UvsY. As shown in Figure 52, products of the correct size were amplified in the presence of UvsY. In the absence of UvsY, with the exception of the MS2 downRT2 + MS2 up5 reaction products, the most abundant product appeared to be of the same size as that synthesised when UvsY is present. It was concluded that, with the template and primer pairs used, RPA DNA amplification is possible in the absence of UvsY and that such reactions often produce products of the correct size.

An additional experiment was carried out to explore whether the UvsY-independent amplification observed previously would occur using different primer pairs synthesising different sized products. The results for this additional experiment exquisitely show just how effective amplification can be using the T6 H66S recombinase in the absence of UvsY (although kinetics are not investigated). General reaction conditions were the same as described in for the experiment depicted in Figure 52, with the following exceptions: reactions were carried out using primer MS2 down5 with primer MS2 up5, up6, up7 or up2. Reactions were also carried out using primers MS2 down2 and MS2 up4. Amplification products were made when using any of the primer combinations and in both presence and absence of UvsY. As shown in Figure 53, all reactions worked well except with the MS2 down5/up5 primer pair, although this still produced a small amount of the correct product. The major product from each reaction was of the correct size regardless of whether UvsY

was present in the reaction or not. In the absence of UvsY there appeared to be a greater abundance of incorrect products, however these were present in lower amounts than the correct product. It was concluded that different sized RPA products can be amplified using a variety of primer pairs and that the ability of the reaction to proceed in the absence of UvsY is unlikely to be dependent upon the primers used or the resultant product size.

UvsY-free amplification of small genomic DNA targets

A study was performed to investigate whether, in the absence of UvsY, the size of the DNA target plays a role in the ability of RPA to amplify DNA. To this end, a small 305bp RPA product, amplified from human genomic DNA, was used as the DNA target in an RPA reaction. Reaction conditions were the same as stated for the experiment depicted in Figure 52, with the exception that the reactions were carried out using 1000 copies of DNA target with primer ApoB4 and either primer ApoB300, ApoB3, ApoB7 or ApoB10, which generate products of 305bp, 210bp, 143bp and 141bp, respectively. As shown in Figure 54, in the absence of UvsY all of the reactions generated DNA amplicons, however despite an apparently robust capability to synthesise DNA products in the absence of UvsY, products generated using T6 H66S UvsX without UvsY were not always those of the expected size and the same size as those produced in the presence of UvsY. Presumably primer-related artefacts are sometimes dominant to bona fide product formation, although the reasons are unclear. It was concluded that in the absence of UvsY, DNA amplification occurs reasonably proficiently using a small DNA target but unlike when UvsY is present, the product is always of the correct size.

UvsY-free amplification of complex genomic targets

This experiment addressed whether low copy numbers of complex genomic targets may be amplified in the absence of UvsY. Reaction conditions were the same as described for the experiment depicted in Figure 52, with the exception that reactions were carried out using 1000 copies of human genomic DNA with primer ApoB4 and either primer ApoB300, ApoB3, ApoB7 or ApoB10, which generate products of 305bp, 210bp, 143bp and 141bp, respectively. As shown in Figure 55, in the absence of UvsY, DNA amplification occurred for all reactions, however the products generated using T6 H66S UvsX without UvsY were not always those of the expected size and the same size as those produced in the presence of UvsY. It was concluded that in the absence of UvsY, DNA amplification occurs efficiently using a complex genomic DNA target but unlike reactions performed in the presence of

UvsY, where the correct product is usually synthesised, the product is always of the correct size.

UvsY free DNA amplification requires PEG

An experiment was performed to address whether the UvsY-independent behaviour exhibited by T6 H66S recombinase extended further to a lack of requirement for PEG. These reactions were conducted as described for the experiment depicted in Figure 52, with the following exceptions: reactions were performed using 1000 copies of human genomic DNA and primer ApoB4 with either primer ApoB300 or ApoB3, both with and without the presence of PEG. As shown in Figure 56, the results demonstrated a stark difference in reaction productivity between when PEG is present or absent. This experiment demonstrated the criticality of the use of polyethylene glycol inclusion in RPA reactions to permit effective amplification. In the absence of PEG, amplification of bona fide products generally does not occur, although a very faint artifact may be present in one lane, perhaps indicating a low level of loaded filaments when the T6 H66S recombinase is employed (although this does not occur in the presence of UvsY). It was concluded that for correct and effective amplification of target DNA, regardless of the presence or absence of UvsY, PEG is necessary in the reaction.

UvsY-Free DNA amplification using T4 gp32 with T6 H66S recombinase

This experiment was performed to investigate whether the UvsY-independent amplification would occur when T4 gp32 was used together with T6 H66S UvsX. The general reaction conditions were as described for the experiment depicted in Figure 52, except that here reactions were conducted using either Rb69 gp32 or 337.5ng/μl T4 gp32. Where T4 gp32 was used in the presence of UvsY, 30ng/μl T4 UvsY was used. 1000 copies of human genomic DNA were used per reaction in conjunction with primer ApoB4 and either primer ApoB300 or ApoB3. As shown in Figure 57, this experiment shows that the UvsY-independent activity of T6 H66S recombinase is still found when T4 gp32, rather than Rb69 gp32, is utilized. The production of clean expected products is less efficient than when using Rb69 gp32, however there can be no doubt that large numbers of recombinationally active filaments are present. It was concluded that DNA amplification plainly occurs when using T4 gp32 in reaction, although, in the terms of correct products, this process is less efficient than if Rb69 gp32 is used.

UvsY-Free DNA amplification using T6 H66S and Aehl gp32

This experiment was performed to investigate whether the UvsY-independent amplification would occur when Aeh1 gp32 was used together with T6 H66S UvsX. Reaction conditions were the same as described for the experiment depicted in Figure 52, with the exception that reactions were carried out using 400ng/ μ l Rb69 gp32 or 360ng/ μ l Aeh1 gp32, and 1000 copies human genomic DNA with primer ApoB4 and either primer ApoB300 or ApoB3. As shown in Figure 58, the results demonstrate when UvsY was omitted and T6 H66S was used, Aeh1 gp32 was unable to support RPA in producing a correct product. However, some small amount of amplification did occur. It was concluded that, when combined with T6 H66S, Aeh1 promotes only limited DNA amplification. This data, when combined with the data previously described, suggests that the efficiency of UvsY-independent behaviour of T6 H66S RPA reactions is to some extent dependent on gp32 type.

UvsY-Free DNA Amplification using T4 UvsX

An experiment was performed to investigate whether the presence of UvsY was needed for DNA amplification to occur when using T4 UvsX with Rb69 gp32. These reactions were conducted as described in the experiment depicted in Figure 52, with the following exceptions: reactions were performed using either T6 H66S UvsX or 123.5ng/ μ l T4 UvsX, and 1000 copies of human genomic DNA with primer ApoB4 and either primer ApoB300 or ApoB3. Where T4 UvsX was used with UvsY, 30ng/ μ l T4 UvsY was utilized. As shown in Figure 59, the results demonstrate that in the presence of UvsY, T4 UvsX reactions generate products of the expected size, as when T6 H66S UvsX is used. However, unlike T6 H66S reactions, when UvsY is omitted no amplification products whatsoever are generated. This experiment shows that under the standard conditions employed T4 UvsX, unlike T6 H66S UvsX, is totally dependent on the presence of UvsY protein. This data confirms a large body of earlier evidence, which showed that UvsY and PEG were both obligate components of RPA systems configured with T4 reagents.

An additional experiment was performed to investigate if by using T4 gp32 instead of Rb69 gp32, whether UvsY-deficient T4 UvsX reactions would continue to fail to produce amplification product. The general reaction conditions were as described for the experiment depicted in Figure 52 with the exception that reactions were performed using either Rb69 gp32 with T6 H66S UvsX or 337ng/ μ l T4 gp32 with 123ng/ μ l T4 UvsX. 1000 copies of human genomic DNA were used per reaction in conjunction with primer ApoB4 and either primer ApoB300 or ApoB3. Where T4 gp32 and UvsX were used with UvsY, 30ng/ μ l T4

UvsY was utilized. As shown in Figure 60, the results demonstrate that, similar to that shown previously, in the presence of UvsY, reactions utilizing T4 components generate products of the correct size and the absence of UvsY negates this. This data confirms the conclusion that under the standard conditions, T4 UvsX, unlike T6 H66S UvsX, is totally dependent on the presence of UvsY protein. In this case T4 gp32 was employed as the single-stranded DNA binding protein.

Yet another experiment was performed to investigate the requirement for UvsY when using T4 UvsX in RPA amplification/detection reactions that utilize a fluorescent probe system to sense DNA accumulation. In this experiment RPA was performed under the following conditions: 100mM Potassium acetate, 50mM Tris.acetate pH 8.3, 14mM Magnesium acetate, 5mM dTT, 200mM dNTPs, 50mM Creatine phosphate (Calbiochem), 2.5mM ATP (Roche), 50ng/μl Creatine Kinase (Roche), amplification primers J1 (120nM) and K2 (480nM), 120nM fluorescent probe BsFlc, 5% PEG 35,000, 43.33ng/μl Sau Polymerase, 600ng/μl Rb69 gp32, 120ng/μl T6 H66S UvsX and 79ng/μl Rb69 UvsY, where appropriate. Nfo was included at 100ng/μl. Samples contained either water or 200 copies of B.subtilis genomic DNA, and were either in the presence or absence of Rb69 UvsY. Reactions were established on ice in a 384-well plate, and then transferred to a BIOTEK Flx-800 fluorescence microplate reader with stage set to 38°C at which time measurements were taken periodically from a bottom-reading probe.

As shown in Figure 61, signal accumulated in a template-dependent manner in reactions configured with T6 H66S recombinase with or without UvsY, and also with reactions containing T4 UvsX in the presence of UvsY. However, in the absence of UvsY, T4 UvsX reactions displayed no DNA amplification capability. It was concluded that under these standard conditions, for DNA amplification to occur, unlike T6 H66S UvsX, T4 UvsX has a strict requirement for UvsY.

An additional experiment was carried out in order to investigate the effects of titrating Rb69 gp32 concentrations on the requirement of T4 UvsX for UvsY. These reactions were conducted as described for the experiment depicted in Figure 61, with the exception that reactions were performed using amplification primers Scii35IV (480 nM) and OrfX45a (120nM), 120nM fluorescent probe SA Tamra2, 125ng/μl T4 UvsX and 30ng/μl T4 UvsY, where appropriate. Rb69 gp32 was used at 400ng/μl, 600ng/μl or 800ng/μl. Samples contained either water or 200 copies of MRSA I genomic DNA, and were either in the

presence or absence of Rb69 UvsY. As shown in Figure 69, DNA amplification occurred in all template samples containing UvsY, regardless of the concentration of Rb69 used. No template sample demonstrated DNA amplification when UvsY was missing. It was concluded that under the standard conditions employed, for DNA amplification to occur, the T4 UvsX protein is dependent on UvsY and that this dependency is not altered by variation of gp32 concentration.

Yet another experiment was carried out to further investigate the requirement of the T4 UvsX protein for UvsY. The reaction conditions used were the same as described for the experiment depicted in Figure 61, with the following exceptions: reactions were performed using amplification primers Sciii (480nM) and OrfX45a (120nM), 120nM fluorescent probe BsFlc beta, 123.5ng/μl T4 UvsX, 500ng/μl Rb69 gp32 and 18ng/μl Sau Polymerase. Samples contained either water or 10000 copies of 506bp PCR DNA fragment, and were either in the presence or absence of Rb69 UvsY. As shown in Figure 70, under these conditions, T4 UvsX reactions efficiently amplify DNA, both in the presence and absence of UvsY. However, DNA amplification in samples that contained UvsY preceded those where UvsY was missing, and at experiment termination more DNA had been amplified in the presence of UvsY than the absence of UvsY. It was concluded that depending upon the conditions employed, T4 UvsX may or may not require the presence of UvsY for DNA amplification to occur. However, even where the conditions allow amplification to occur in the absence of UvsY, the addition of UvsY improves the reaction rate and increases the amplified DNA output.

To further elucidate the requirement of T4 UvsX protein for UvsY, a further experiment was performed. The reaction conditions and samples were those described in Figure 70. Following reaction completion, each of the samples was purified using the GenElute PCR cleanup kit (Sigma) and visualized using gel electrophoresis. As shown in Figure 71, gel electrophoresis can be used as an additional method (process) of visualizing the data collected for DNA amplification using RPA, such as the experiment described in Figure 70. The results shown in Figure 71 further demonstrate that under these conditions, T4 UvsX enables DNA amplification to occur both in the presence and absence of UvsY. However, as described for the experiment depicted in Figure 70, more DNA was amplified in the presence of UvsY than in the absence of UvsY. These results confirm that under certain

conditions, T4 UvsX can support DNA amplification in the absence of UvsY, however the amount of DNA amplification is improved in the presence of UvsY.

UvsY-Free DNA Amplification using T6 UvsX

This experiment was performed to determine whether the unmodified T6 UvsX protein exhibits the capacity to amplify DNA in the absence of UvsY. The reaction conditions used were as described for the experiment depicted in Figure 52, with the exception that the reactions were performed using T6 H66S UvsX or 120ng/μl T6 UvsX, and 1000 copies of human genomic DNA with primer ApoB4 and either primer ApoB300 or ApoB3. As shown in Figure 62, one of the two amplicons examined was efficiently amplified, while one was not, in the absence of UvsY. Furthermore, the relative efficiency of amplification of fragments between T6 and T6 H66S recombinase with or without UvsY were variant. While one cannot exclude preparation-dependent variations in between the recombinase proteins, this data is consistent with the suggestion that the unmodified and modified recombinase demonstrate variant activities as indicated earlier. It was concluded that in the absence of UvsY, DNA amplification can occur with T6 UvsX, although the efficiency to do this is different from T6 H66S UvsX.

UvsY-Free DNA amplification using Rb69 UvsX

This experiment investigated whether Rb69 UvsX requires UvsY for efficient amplification. The reactions were conducted as described for the experiment depicted in Figure 52, with the exception that the reactions were performed using T6 H66S UvsX or 120ng/μl Rb69 UvsX, 400ng/μl Rb69 gp32, and 1000 copies of human genomic DNA with primer ApoB4 and either primer ApoB300 or ApoB3. As shown in Figure 63, no amplification was seen in the absence of UvsY consistent with a strict dependence on the presence of UvsY under the conditions employed. Even with UvsY present, amplification was poor, so some caution should be placed on the interpretation. Without intending to be bound by any theory, the simplest explanation is that like with T4 UvsX, UvsY is required to achieve filament-loading levels required for effective and sensitive amplification. It was concluded that under the standard conditions employed, Rb69 UvsX is likely to require UvsY for efficient DNA amplification to be achieved.

UvsY-Free DNA amplification using Aeh1 UvsX

This experiment was performed to address whether Aeh1 UvsX requires UvsY for efficient amplification. Reaction conditions were as described for the experiment depicted in

Figure 52, with the exception that the reactions were carried out using 400ng/μl Rb69 gp32 for reactions with T6 H66S UvsX, and for reactions with Aeh1: 500ng/μl Rb69 gp32 UvsX, 200ng/μl Aeh1 UvsX and 80ng/μl Aeh1 UvsY, where UvsY was included. 1000 copies of human genomic DNA were used per reaction in conjunction with primer ApoB4 and either primer ApoB300 or ApoB3. As shown in Figure 64, no amplification was seen in the absence of UvsY where Aeh1 proteins were used, while with the presence of UvsY, correct sized products were evident. It was concluded that Aeh1 proteins are unable to undergo DNA amplification in the absence of UvsY, consistent with a strict dependence on the presence of UvsY under the conditions employed.

10 *UvsY-Free DNA Amplification using modified Rb69 UvsX (T6 DNA binding loop2, modification of histidine 64 to serine, and modified C terminus (LDEx2))*

This experiment was performed to investigate whether modified Rb69 UvsX containing the DNA binding loop2 of T6UvsX requires UvsY for efficient amplification, and to determine whether the variant DNA binding loop2 of T6 recombinase accounted for the UvsY-independent activity of T6 recombinases. Reaction conditions employed were as described for the experiment depicted in Figure 52, with the following exceptions: reactions were performed using 400ng/μl Rb69 gp32 and either T6 H66S UvsX or 120ng/μl T6 H64S 2xLDE UvsX. 1000 copies of human genomic DNA were used per reaction in conjunction with primer ApoB4 and either primer ApoB300 or ApoB3. As shown in Figure 65, no amplification was seen in the absence of UvsY consistent with dependence on the presence of UvsY under the conditions employed.

Without intending to be bound by any theory, one interpretation of this experiment is that the DNA binding loop 2, in isolation, may be insufficient to confer UvsY-independent activity on hybrid recombinases. However caution should be exercised as poor amplification was observed with this protein even in the presence of UvsY. It was concluded that, under the standard conditions employed, T6 H64S 2xLDE UvsX is likely to require UvsY for efficient DNA amplification to be achieved.

EXAMPLE 16: gp32 Activity

The ability to measure the effectiveness of gp32 in regulating the cutting rate proves to be a very accurate approach to assess gp32 activity, something which has been historically difficult to assess. An experiment was performed to demonstrate a useful assay for the activity of gp32 preparations. Experimental conditions were as follows: reactions were

performed in 50 µl volume; final concentration of probe (SA-Tamra2; 5'-tggttaattgagcaagtgtatagagcatraygabtatgcgtggag-3' (SEQ ID NO:125), here y= thf, b= BHQ2-dT, r= TAMRA-dT, 3'= Bio-TEG) was 100nM; Rb69 gp32 was used at final concentration of 0, 40, 50, 63, 83, 100, 125, 167, or 250 ng/µl; Nfo was present at 33ng/ µl; buffer conditions
 5 were 20 mM Tris-acetate, 50 mM potassium acetate (pH 7.9), 10 mM Magnesium Acetate, 1 mM Dithiothreitol.

As depicted in Figure 66, the results of this experiment show that a single-stranded probe which contains a fluorophore and quencher separated by a tetrahydrofuran residue (THF) can be rapidly cut by an excess of Nfo nuclease when present in an aqueous buffered
 10 solution, and in the absence of gp32 protein. This activity was robust under these conditions despite claims in the literature that Nfo targets only duplex DNA substrates. Without intending to be bound by any theory, the activity may arise by formation of transient duplex structures, hairpins and the like, under the conditions used here. This activity was entirely suppressed by an excess of gp32 protein when included in the reaction mixture. When the
 15 mass of gp32 was progressively decreased, cutting activity was once again detected (monitored by increasing fluorescence over time) and the rate of cutting was regulated by the mass of gp32 added at these limiting final concentrations. Furthermore, by setting the concentration of gp32 at limiting levels in experiments it was possible to assess consequences of a variety of manipulations such as the effects of competitor nucleic acids or temperature on
 20 gp32 behaviour and turnover.

Biochemical distinction among different species of gp32 molecules

An experiment was performed to assess whether gp32 molecules from different species of origin were biochemically distinct from one another. Experimental conditions were as follows: reactions were performed in 50 µl volume; final concentration of probe (SA-
 25 Tamra2; 5'-tggttaattgagcaagtgtatagagcatraygabtatgcgtggag-3' (SEQ ID NO:126), here y= thf, b= BHQ2-dT, r= TAMRA-dT, 3'= Bio-TEG) was 100nM; Rb69 gp32 was used at final concentration of 80 ng/µl, Nfo was present at 33 ng/µl; after 350 sec either water, dsDNA (550 ng human genomic DNA; i.e. approximately 10x mass of oligonucleotide probe) or ssDNA (28 pmol oligonucleotide of sequence 5'ctgtattagaagtacatgctgatcaagtgaca-3' (SEQ ID
 30 NO:127)) was added; buffer conditions were 20 mM Tris-acetate, 50 mM potassium acetate (pH 7.9), 10 mM Magnesium Acetate, 1 mM Dithiothreitol. Measurements were made in a

fluorometer manufactured by Embedded System Engineering (ESE,GmbH) with LED excitation of 520nm and emission at 585 nm.

Initially, a concentration of gp32 was established which permitted only a very low cutting activity by Nfo nuclease on the fluorophore/quencher probe. At this concentration the availability of gp32 was limited to a minimal quantity for substantial probe protection, and under these conditions it was possible to assess very sensitively whether gp32 was partitioned away from the probe in competition experiments. After monitoring the slow probe cutting for a while, excess double-stranded DNA or single-stranded oligonucleotides were added to assess whether this affected the distribution of gp32 on the probe. In all cases the addition of excess in single-stranded oligonucleotide lead to a sudden and pronounced increase in fluorescence and hence probe cutting. However, rather interestingly, it was discovered that T4 gp32 was strongly influenced by the addition of the duplex DNA as cutting became very pronounced indicating loss of gp32 from the probe DNA, while Rb69 and Aeh1 gp32 species showed only slight increases in cutting. Clearly Rb69 and Aeh1 gp32 molecules differentiated and partitioned much more effectively in favour of single-stranded DNA than T4 gp32. The results are depicted in Figure 67, which shows that T4 and Rb69 gp32 molecules are biochemically distinct in regard to partitioning between single-stranded and duplex DNAs.

20

Temperature limits for different gp32 species

An experiment was performed using the probe protection assay to assess at what upper temperature different species of gp32 failed to function correctly. Experimental conditions were as follows: reactions were performed in 50 µl volume; final concentration of probe (SA-Tamra2; 5'-tgtaattgagcaagtgtatagagcatraygabtatgcgtggag-3' (SEQ ID NO:128), where y= thf, b= BHQ2-dT, r= TAMRA-dT, 3'= Bio-TEG) was 100 nM; Rb69 gp32 was used at final concentration of 80 ng/µl, Nfo was present at 33 ng/µl; after 350 sec the temperature was gradually raised (see graph); buffer conditions were 20 mM Tris-acetate, 50 mM potassium acetate (pH 7.9), 10 mM Magnesium Acetate, 1 mM Dithiothreitol. Measurements were made in a fluorometer manufactured by Embedded System Engineering (ESE,GmbH) with LED excitation of 520 nm and emission at 585 nm.

Concentrations of gp32 were used that lead to a situation in which gp32 were just limiting with regard to probe protection. Reactions were then continuously monitored after a heat source was applied such that temperature gradually increased in the reaction environment. Indicated temperatures referred to those read from a thermocouple in the fluorescent probe device utilized which was close to the tube containing the reaction and thus a good indicator of the reaction temperature. As shown in Figure 68, there were differences in upper temperature activity limits for different gp32 species. As the temperature rises the slope of the fluorescence curves initially remained constant, but at some point began to increase. Without intending to be bound by any theory, this evidence indicated that the gp32 was losing its effectiveness because the protein was becoming structurally unstable. Support for this interpretation rather than an interpretation in which Nfo activity progressively increases, is provided by the fact that T4 gp32 does not show any rate changes until relatively high temperatures, while when other gp32 molecules are used, changes begin much earlier. In particular it was noted that Aeh1 gp32 became highly ineffective at about 40°C, and displayed a pronounced loss of activity in the assay above this point. Rb69 gp32 also appeared to be less tolerant of higher temperatures than T4 gp32 and became partially affected by about 42°C. T4 gp32 is much more resistant and was still functioning at a temperature of at least 47°C.

The data describes herein supports the discovery of novel, diverse, hybrid and engineered recombinase enzymes and the utility of such enzymes for carrying out RPA reactions. The data further supports the identification of optimal conditions for carrying out RPA reactions using the novel, diverse, hybrid and engineered recombinase agents described herein and associated recombination factors. While specific embodiments of the subject invention have been discussed, the above specification is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The appendant claims are not intended to claim all such embodiments and variations, and the full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

All publications, patent applications, and patents mentioned herein, including those items listed below, are hereby incorporated by reference in their entireties as if each individual publication or patent was specifically and individually indicated to be incorporated

by reference. In case of conflict, the present application, including any definitions herein, will control.

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We claim:

1. An recombinase polymerase amplification process of amplification of a double stranded target nucleic acid molecule, comprising the steps of:
 - (a) contacting UvsX, UvsY, and gp32 proteins with a first and a second single stranded nucleic acid primer specific for said double stranded target nucleic acid molecule to form a first and a second nucleoprotein primer, wherein said UvsX, UvsY, and gp32 are each derived from a myoviridae phage, and wherein no more than two of said UvsX, UvsY and gp32 proteins are T4 phage proteins;
 - (b) contacting the first nucleoprotein primer to said double stranded target nucleic acid molecule to create a first D loop structure at a first portion of said double stranded target nucleic acid molecule and contacting the second nucleoprotein primer to said double stranded target nucleic acid molecule to create a second D loop structure at a second portion of said double stranded target nucleic acid molecule such that the 3' ends of said first nucleic acid primer and said second nucleic acid primer are oriented toward each other on the same double stranded target nucleic acid molecule without completely denaturing the target nucleic acid molecule;
 - (c) extending the 3' end of said first and second nucleoprotein primer with one or more polymerases capable of strand displacement synthesis and dNTPs to generate a first and second double stranded target nucleic acid molecule and a first and second displaced strand of nucleic acid ; and
 - (d) continuing the reaction through repetition of (b) and (c) until a desired degree of amplification is reached.
2. The process of claim 1 wherein said first and second displaced strand of nucleic acid hybridizes to each other after step (c) to form a third double stranded target nucleic acid molecule.
3. The process according to claim 1 in which the myoviridae phage from which the UvsX, UvsY and gp32 proteins are derived is selected from the group consisting of: T4, T2, T6, Rb69, Aeh1, KVP40, Acinetobacter phage 133, Aeromonas phage 65, cyanophage P-SSM2, cyanophage PSSM4, cyanophage S-PM2, Rb14, Rb32, Aeromonas phage 25,

Vibrio phage nt-1, phi-1, Rb16, Rb43, Phage 31, phage 44RR2.8t, Rb49, phage Rb3, and phage LZ2.

4. The process according to claim 1 wherein said UvsX, UvsY and gp32 are selected from the group consisting of:

- 5 (a) Rb69 UvsX, Rb69 UvsY and Rb69 gp32;
 (b) Aeh1 UvsX, Aeh1 UvsY and Rb69 gp32;
 (c) T4 UvsX, T4 UvsY and Rb69 gp32; and
 (d) T4 UvsX, Rb69 UvsY and T4 gp32.

5. The process according claim 1 wherein said UvsX, UvsY, and gp32 are native, hybrid or mutant proteins from the same or different myoviridae phage sources.

6. The process according to claim 5, wherein said hybrid protein comprises one or more amino acid residues from two different species of myoviridae phages to yield a protein with improved performance characteristics in said process.

7. The process according to claim 5, wherein said UvsX is a mutant UvsX.

8. The process according to claim 7, wherein the mutant UvsX is an Rb69 UvsX comprising at least one mutation in the Rb69 UvsX amino acid sequence, wherein the mutation is selected from the group consisting of:

an amino acid which is not histidine at position 64;

a serine at position 64;

the addition of one or more glutamic acid residues at the C-terminus;

the addition of one or more aspartic acid residues at the C-terminus; and

a combination thereof.

9. The process according to claim 7, wherein the mutant UvsX is a T6 UvsX having at least one mutation in the T6 UvsX amino acid sequence, wherein the mutation is selected from the group consisting of:

an amino acid which is not histidine at position 66;

a serine at position 66;

the addition of one or more glutamic acid residues at the C-terminus;

the addition of one or more aspartic acid residues at the C-terminus; and

a combination thereof.

10. The process according to claim 6, wherein said hybrid protein is a UvsX protein comprising at least one region which comprises an amino acid sequence from a different UvsX species.
11. The process according to claim 10, wherein said at least one region is the DNA-binding loop-2 region of UvsX.
12. The process according to claim 1, wherein said process is performed in the presence of a crowding agent selected from the group comprising polyethylene glycol, polyethylene oxide, polyvinyl alcohol, polystyrene, Ficoll, dextran, PVP, albumin.
13. The process according to claim 12, wherein the crowding agent has a molecular weight of less than 200,000.
14. The process according to claim 12, wherein the crowding agent is present in an amount of about 0.5% to about 15% w/v.
15. The process according to claim 1, wherein the polymerase is a large fragment polymerase selected from the group consisting of E.Coli Pol I, Bacillus subtilis Pol I, Staphylococcus aureus Pol I, and homologues thereof.
16. The process according to claim 1, wherein said process is performed in the presence of heparin.
17. The process according to claim 1, wherein said first or second nucleic acid primers is a blocked primer, and wherein said process is performed in the presence of an endonuclease selected from the group consisting of E.coli exonuclease III and E.coli endonuclease IV.
18. The process according to claim 1 wherein said process is performed in the presence of about 1 mM to about 8 mM divalent manganese ions.
19. The process according to claim 1, wherein said process is performed in the absence of UvsY.
20. The process of claim 1 wherein at least one of said UvsX, UvsY or gp32 protein comprises an amino acid sequence selected from the group consisting of: SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, SEQ ID NO:119, SEQ ID NO:120, SEQ ID NO:121, SEQ ID NO:122, SEQ ID NO:123, and SEQ ID NO:124.

21. An recombinase polymerase amplification process of amplification of a double stranded target nucleic acid molecule with a first and a second strand of DNA, comprising the steps of:

- (a) contacting UvsX, UvsY, and gp32 proteins with a first single stranded nucleic acid primer specific for said double stranded target nucleic acid molecule to form a population of first nucleoprotein primer, wherein said UvsX, UvsY, and gp32 are each derived from a myoviridae phage, and wherein no more than two of said UvsX, UvsY and gp32 proteins are T4 phage proteins;
- (b) contacting the first nucleoprotein primer with said double stranded target nucleic acid molecule thereby forming a first D loop structure at a first portion of said double stranded target nucleic acid molecule without completely denaturing the target nucleic acid molecule;
- (c) extending the 3' end of said first nucleoprotein primer with one or more polymerases capable of strand displacement synthesis and dNTPs to generate a double stranded target nucleic acid molecule and a displaced strand of nucleic acid molecule;
- (d) hybridizing a second single stranded nucleic acid primer with said displaced strand of nucleic acid molecule to form a hybridized second single stranded nucleic acid primer;
- (e) elongating said hybridized second single stranded nucleic acid primer to generate a double stranded target nucleic acid molecule;
- (f) continuing the reaction through repetition of (b) and (e) until a desired degree of amplification is reached.

22. A mutant or hybrid Rb69 UvsX protein comprising an alteration in the wildtype Rb69 UvsX amino acid sequence, wherein the alteration in the wildtype amino acid sequence is selected from the group consisting of :

- an amino acid which is not histidine at position 64;
- a serine at position 64;
- the addition of one or more glutamic acid residues at the C-terminus;
- the addition of one or more aspartic acid residues at the C-terminus;

the replacement of DNA-binding loop-2 region with a DNA-binding loop-2 region from a UvsX protein which is not Rb69 UvsX;
the addition of a histidine tag; and
a combination thereof.

- 5 23. The mutant or hybrid Rb69 UvsX protein of claim 22 wherein said protein comprises an amino acid sequence of SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, SEQ ID NO:119, SEQ ID NO:121, or SEQ ID NO:122.
24. A mutant or hybrid T6 UvsX protein comprising an alteration in the wildtype T6 UvsX amino acid sequence, wherein the alteration in the wildtype amino acid sequence is
10 selected from the group consisting of:
an amino acid which is not histidine at position 66;
a serine at position 66;
a valine at position 164;
a serine at position 166;
15 the addition of one or more glutamic acid residues at the C-terminus;
the addition of one or more aspartic acid residues at the C-terminus;
the replacement of DNA-binding loop-2 region with a DNA-binding loop-2 region from a UvsX protein which is not T6 UvsX;
the addition of a histidine tag and
20 a combination thereof.
25. The mutant or hybrid T6 UvsX protein of claim 24 wherein said protein comprises an amino acid sequence of SEQ ID NO:105 or SEQ ID NO:106.

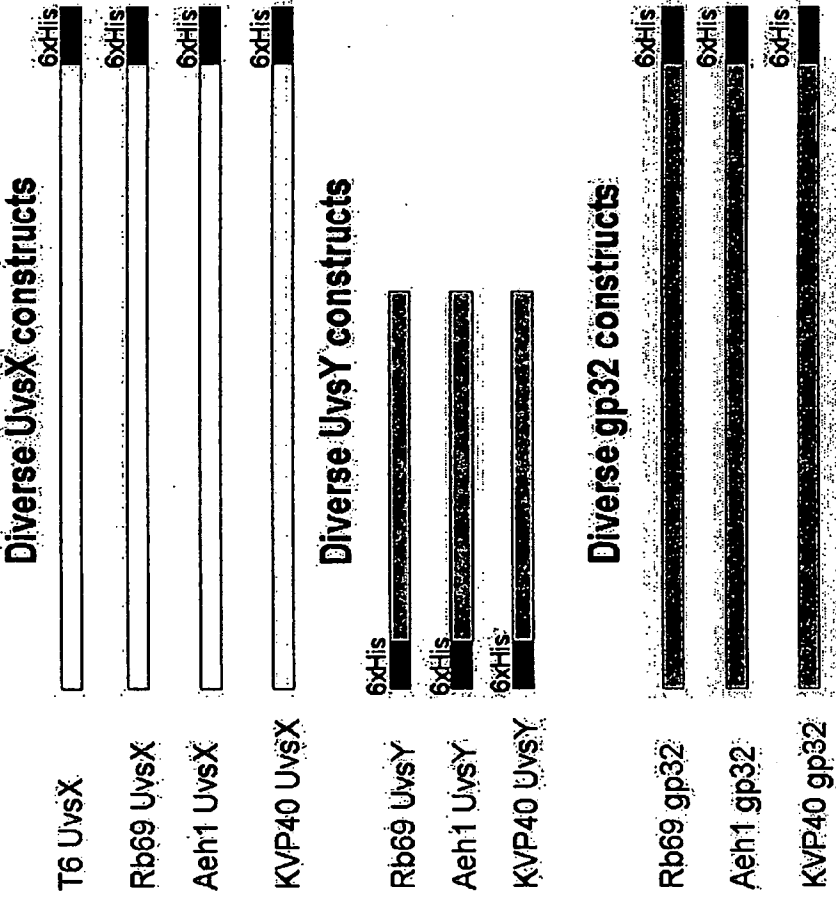
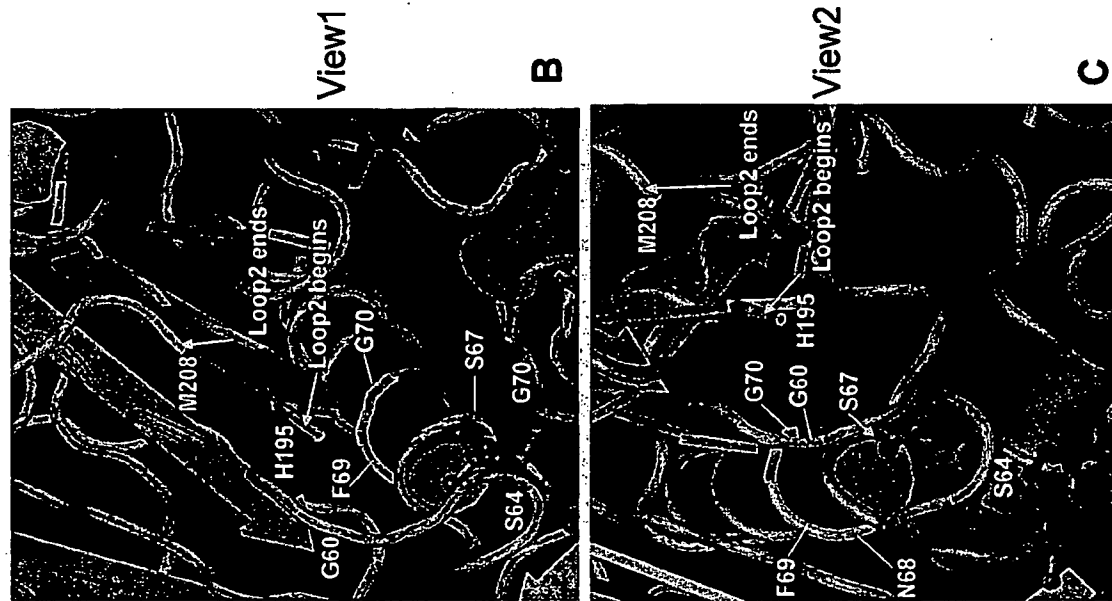


Fig. 1

Alignment of T4 UvsX and E.coli recA

T4	MS--DLKSRLLKASTSKLTAEILTASKEFN-----EKDVVRKIPMMNIALSGEITGGM	
Coli	MAIDENKQKALAAALGQLEKQEGKGSINELGEDRSMVDVETISGSLSIDAIG---AGGL	
	*: : * : : * : : * : : * : : * : : * : : * : : * : : * : : * : : *	
	Walker A motif	
T4	QSGLLI-LAGPSKSEKSNFGLTWSSVNRQYDPDAVCLEYSDFGTPAYLRSMGVDERV	
Coli	PMGRIVEIINGPSSGKTLITLQVTAQAQR--GKTCAFIDAEHLDPPIYARKKGVDDNL	
	* : : * : : * : : * : : * : : * : : * : : * : : * : : * : : * : : *	
	DNA binding loop1	
T4	IHTPVQSLEQLREDMNQLDATERGERV-VVEIDSLGNLASKKETEDALNEKVASDMTRA	
Coli	LCSQPDTEQO----ALEICDALARGAVDIVVDSVAALTPKAEIEGEIGDSHMG--LAA	
	: : : * : : * : : * : : * : : * : : * : : * : : * : : * : : *	
	DNA binding loop2	
T4	KTMKSLFRIVTPYESTKNIPCIALNHT-YETQEMFS--KTMVGGSTGPMYSADTVFIGK	
Coli	RNMSQAMRKLAGNLRQSNTLLIFINQIRMKIGVMEGNPETTTGGNALKFYASVRLDIRRI	
	: * : : * : : * : : * : : * : : * : : * : : * : : * : : * : : *	
T4	ROIKDGSDIQGYQFVNLVEKSRTVKEKSEFFIDVKFDGGIDPYSGLLDMALELGFVVPK	
Coli	GAVTEGENAVGSETRVWKNKIAPEKQAEFFQLLYGEGINFYGEIVDIGVKEKLEK-A	
	: * : : * : : * : : * : : * : : * : : * : : * : : * : : * : : *	
T4	NGWYAREFIDEETGEMTREKSWRAKDTNCTTFMGPIFKHQPFRDAIKRAYQLGALDSNE	
Coli	GAWYSYK-----GEKIGQCKA-----NATAM--LKONP-----ETAK	
	: * : : * : : * : : * : : * : : * : : * : : * : : * : : * : : *	
T4	IVEAEVDEILINSKVTEKSPESKSKSAADLETDLQLSDMEETNE	
Coli	ETKKAVRELLLS-----NPNSTPDFSVD--DSEGVAETNE-DF	
	: * : : * : : * : : * : : * : : * : : * : : * : : * : : * : : *	

Fig. 2



A
***E. coli* recA model (Vanlook MS et al. 2002)**

Structure images generated with CN3D. The structure is recA, however named amino acids refer to possible approximate position of T4 Uvsx amino acids inferred from primary sequence alignment and assumption of concurrent structural conservation.

Fig. 3

Alignment of T4 and T6 gp32

```

t6 MEKRRSTAEIAAOMAKLAGNKGEFSSDKGEWKIKLDNAGNGOAVIRELPSKNDEQAEFA
t4 MEKRRSTAEIAAOMAKLNGNK-GFSSDKGEWKIKLDNAGNGOAVIRELPSKNDEQAEFA
***
t6 ILVNHGFKKNGKWIETCSSTHGDYDSCPVCOYISKNDLYNTDNKEYSLVKRRTSYTANI
t4 ILVNHGFKKNGKWIETCSSTHGDYDSCPVCOYISKNDLYNTDNKEYSLVKRRTSYTANI
***
t6 LVVKDPAAPENEGKVEKYRECKKIWDKINAVADVEMGETPVDVTCPEEGANFVLKVKQ
t4 LVVKDPAAPENEGKVEKYRECKKIWDKINAVADVEMGETPVDVTCPEEGANFVLKVKQ
***
t6 VSGFSNYDESKFLNQSAIENIDDESEOKELFEQNVLDSEMTSKDKKSPFEEISTKE SQVM
t4 VSGFSNYDESKFLNQSAIENIDDESEOKELFEQNVLDSEMTSKDKKSPFEEINTKE SQVM
***
t6 GTAMGGGAATAAKKADKVADDDLDAPNVDDNTKTEDDDESSSGSSSSADDTDEDLLN
t4 GTAMGGGAATAAKKADKVADDDLDAPNVDDNTKTEDDDESSSGSSSSADDTDEDLLN
***
t6 DL
t4 DL
**

```

Alignment of T4 and T6 UvsY

```

t4 MRLEDLQBELKKDVEIDSKLQYEANNVNVLYSKMLNKHSSIKKEMLRIEAOKKVALKAR
t6 MRLEDLQBELKKDVEIDSKLQYEANNVNVLYSKMLNKHSSIKKEMLRIDAOKKVALKAK
***
t4 LDYYSGRGDGEDEFSDRYERSEMKTVLSADKDVLVKVDTSLOYMGHILLDFCSGALDAIKSR
t6 LDYYSGRGDGEDEFSDRYERSEMKTVLSADKDVLVKVDTSLOYMGHILLDFCSGALDAIKSR
***
t4 GFAIKHIQDMRAFEAGK
t6 GFAIKHIQDMRAFEAGK
***

```

Fig. 4

Alignment of UvsX relatives

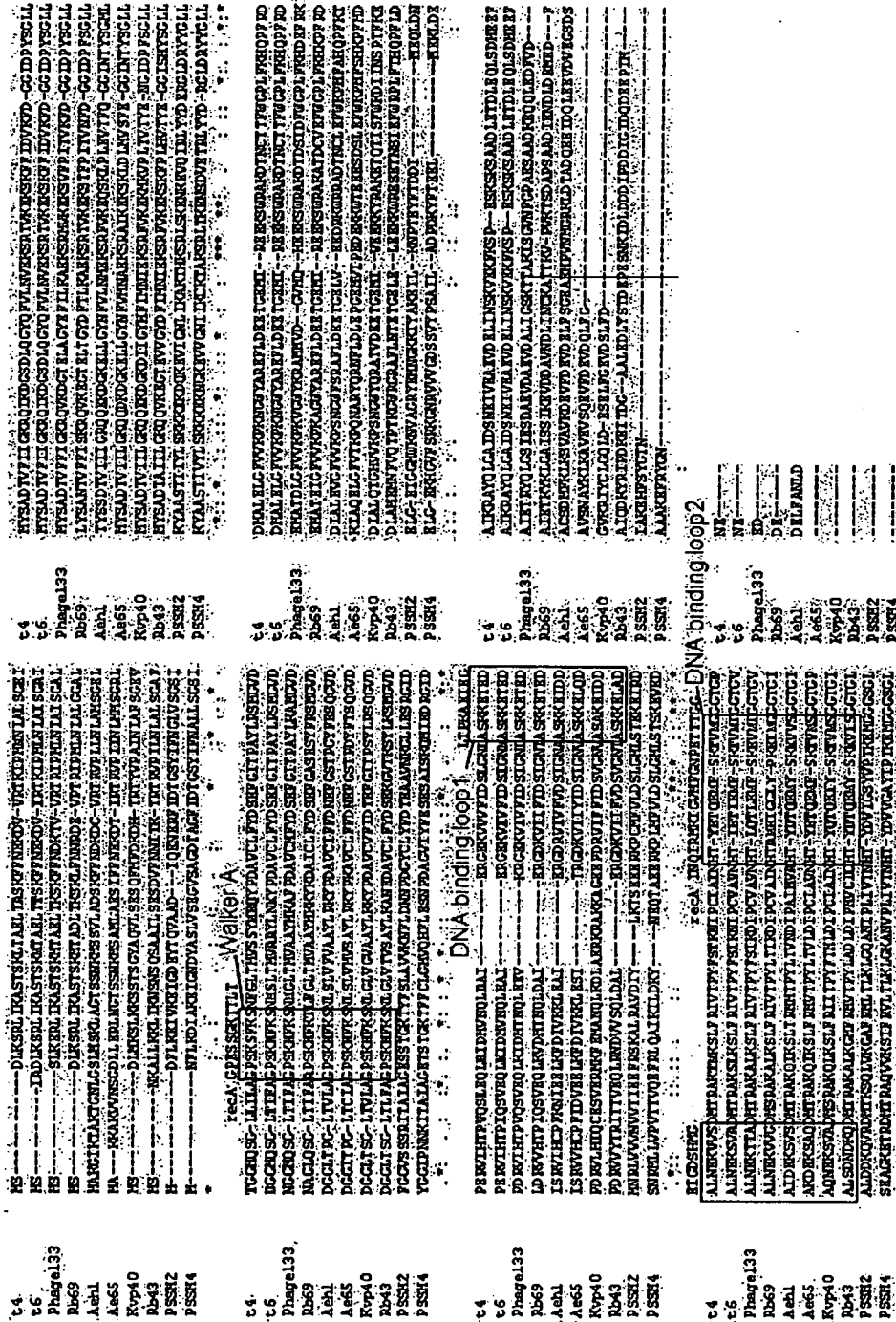


Fig. 5

Alignment of UvsY proteins

t4	MR---	LEDIQEELKRVFIDSTKIQVEAANNVMLYKWKHSHSIKKNLRIEAKKVAL
t6	MR---	LEDIQEELKRVFIDSTKIQVEAANNVMLYKWKHSHSIKKNLRIEAKKVAL
Rb69	MR---	LEDIQEELKRVFIDSTKIQVEAANNVMLYKWKHSHSIKKNLRIEAKKVAL
Phage 133	MK---	LEDIQEELKRVFIDSTKIQVEAANNVMLYKWKHSHSIKKNLRIEAKKVAL
Aeh1	MT---	LEDIQEELKRVFIDSTKIQVEAANNVMLYKWKHSHSIKKNLRIEAKKVAL
Rb43	MT---	LEDIQEELKRVFIDSTKIQVEAANNVMLYKWKHSHSIKKNLRIEAKKVAL
Kvp40	MT---	LEDIQEELKRVFIDSTKIQVEAANNVMLYKWKHSHSIKKNLRIEAKKVAL
PSSM2	MT---	LEDIQEELKRVFIDSTKIQVEAANNVMLYKWKHSHSIKKNLRIEAKKVAL
PSSM4	MT---	LEDIQEELKRVFIDSTKIQVEAANNVMLYKWKHSHSIKKNLRIEAKKVAL
t4	KARLDYYS	SGRGDGEESND-----RYEKS-EMKTVLSADKDVLRKDTSLQYWGIIIDPFC
t6	KARLDYYS	SGRGDGEESND-----RYEKS-EMKTVLSADKDVLRKDTSLQYWGIIIDPFC
Rb69	KARLDYYS	SGRGDGEESND-----RYEKS-EMKTVLSADKDVLRKDTSLQYWGIIIDPFC
Phage 133	KARLDYYS	SGRGDGEESND-----RYEKS-EMKTVLSADKDVLRKDTSLQYWGIIIDPFC
Aeh1	KARLDYYS	SGRGDGEESND-----RYEKS-EMKTVLSADKDVLRKDTSLQYWGIIIDPFC
Rb43	KARLDYYS	SGRGDGEESND-----RYEKS-EMKTVLSADKDVLRKDTSLQYWGIIIDPFC
Kvp40	KARLDYYS	SGRGDGEESND-----RYEKS-EMKTVLSADKDVLRKDTSLQYWGIIIDPFC
PSSM2	KARLDYYS	SGRGDGEESND-----RYEKS-EMKTVLSADKDVLRKDTSLQYWGIIIDPFC
PSSM4	KARLDYYS	SGRGDGEESND-----RYEKS-EMKTVLSADKDVLRKDTSLQYWGIIIDPFC
t4	SGALDAIKSR	SGFALNHIQDMRFEAG-K
t6	SGALDAIKSR	SGFALNHIQDMRFEAG-K
Rb69	SGALDAIKSR	SGFALNHIQDMRFEAG-K
Phage 133	SGALDAIKSR	SGFALNHIQDMRFEAG-K
Aeh1	SGALDAIKSR	SGFALNHIQDMRFEAG-K
Rb43	SGALDAIKSR	SGFALNHIQDMRFEAG-K
Kvp40	SGALDAIKSR	SGFALNHIQDMRFEAG-K
PSSM2	SGALDAIKSR	SGFALNHIQDMRFEAG-K
PSSM4	SGALDAIKSR	SGFALNHIQDMRFEAG-K

Fig. 6

Alignment of gp32 proteins

Clustal (1-10) formatted alignment by MAFFT (v2.0.6)

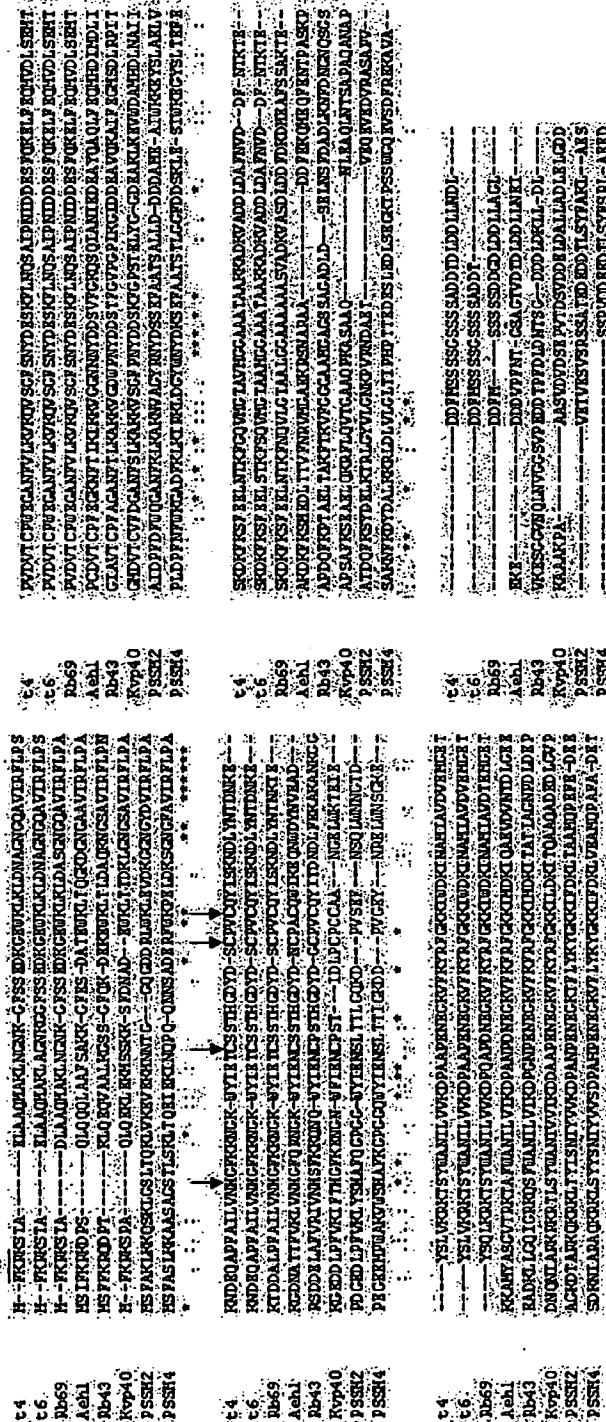


Fig. 7

T6 UvsX may substitute for T4 UvsX in RPA reactions

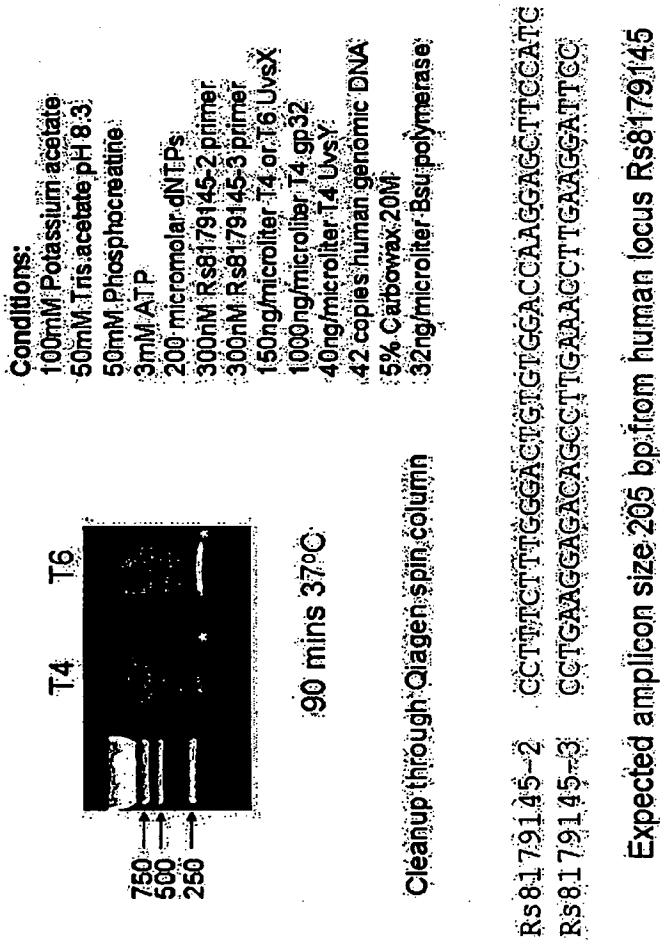


Fig. 8

Comparison of T6 Uvsx protein and T4 Uvsx protein

Key:
T4=Using T4 UvsX
T6=Using T6 UvsX
Numbers of copies of target DNA are indicated

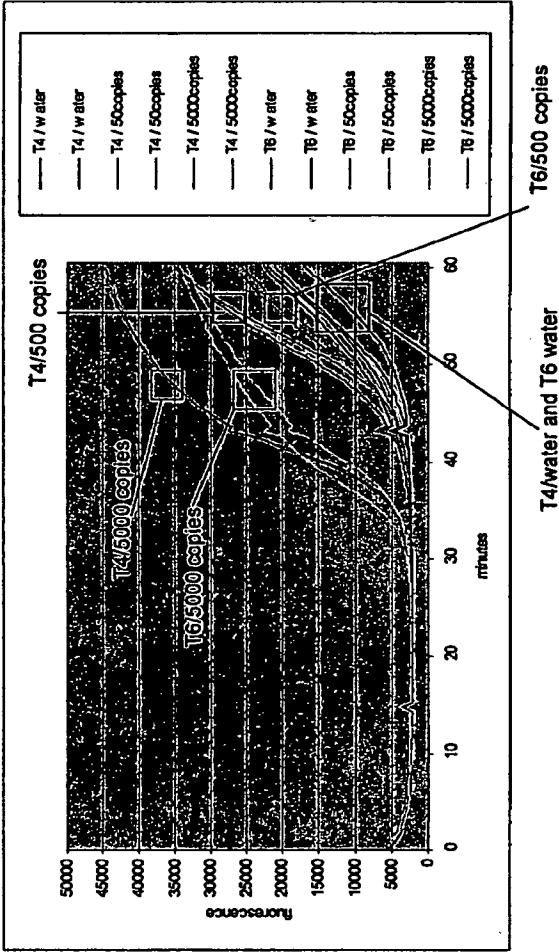
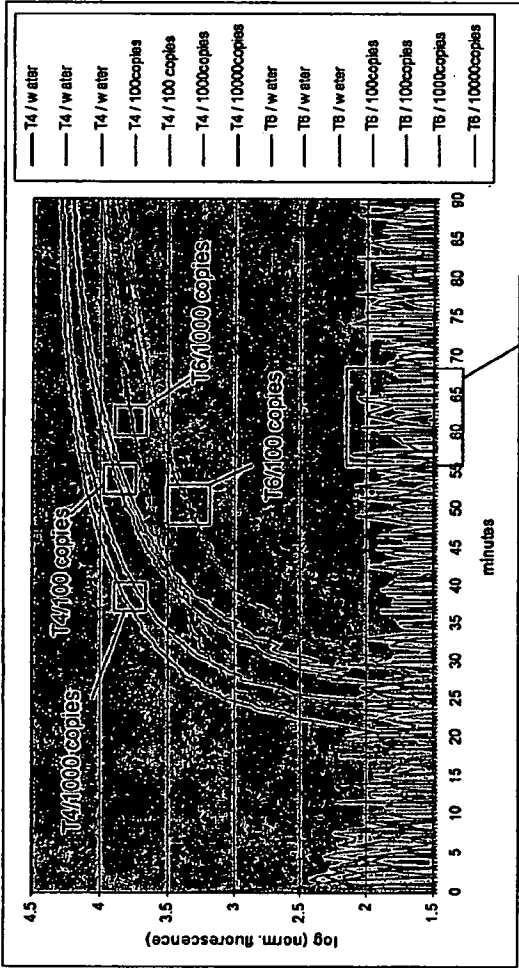


Fig.9

Comparison of T6Uvsx protein and T4Uvsx protein

Key:
T4=Using T4 UvsX
T6=Using T6 UvsX
Numbers of copies of target DNA are indicated



T4/water and T6/water

Fig. 10

T6 Constructs

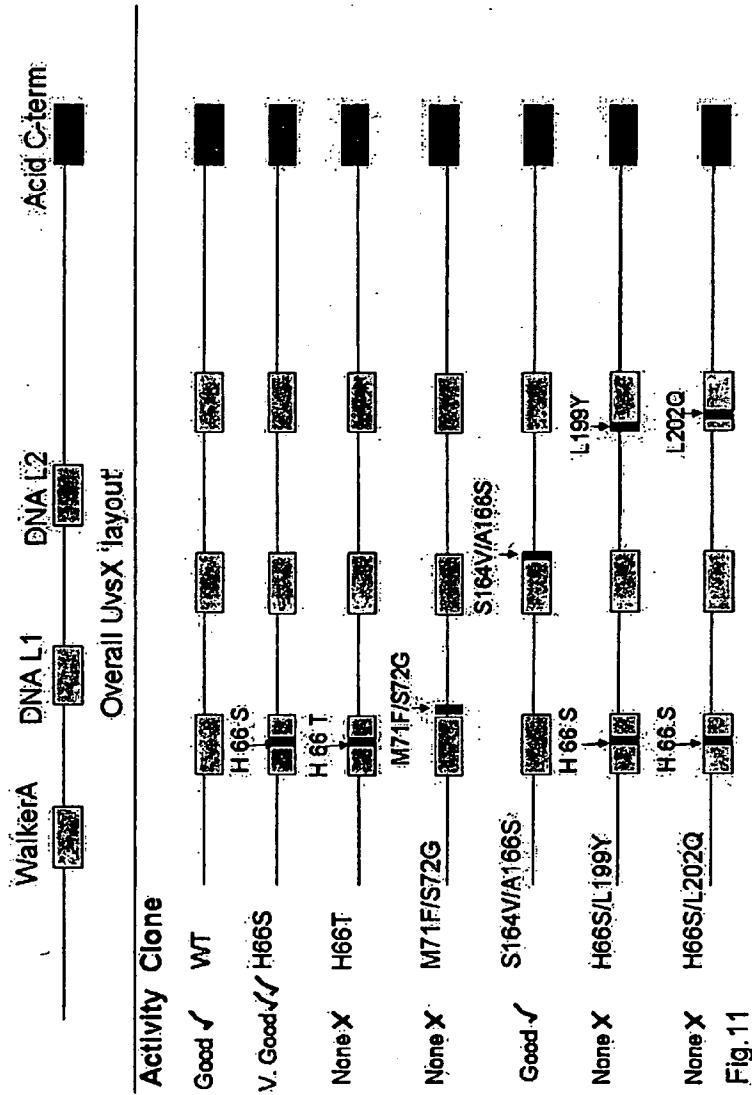


Fig. 11

Comparison of T6 UvsxH66S and T6 UvsXwt

Key:
T6=Using T6 UvsX
T6UvsxH66S=using the T6UvsxH66S protein
Numbers of copies of target DNA are indicated

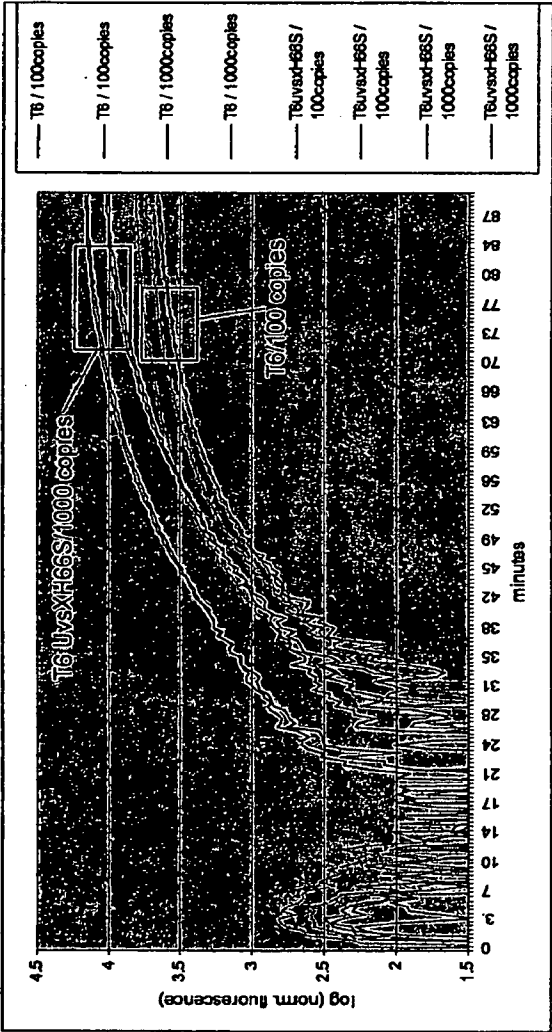
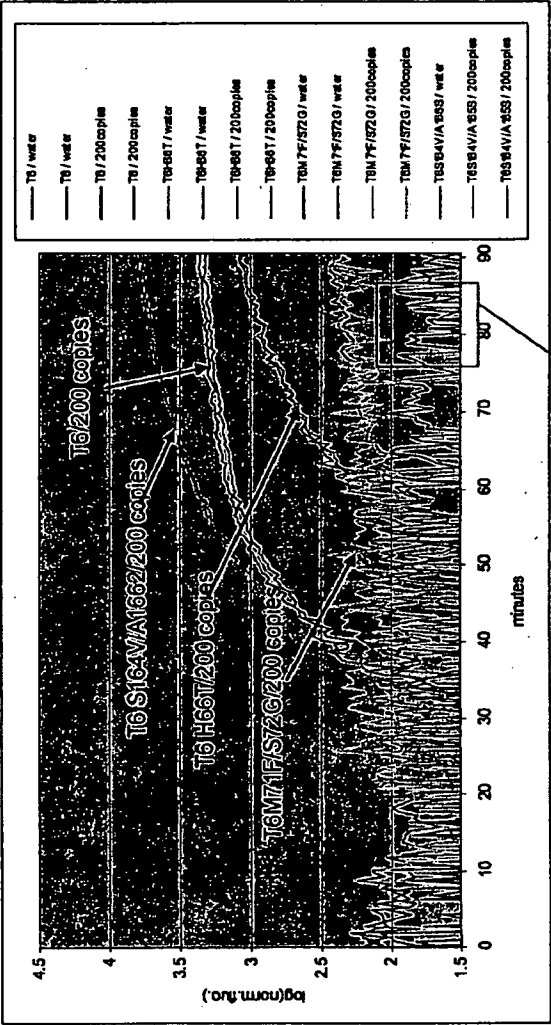


Fig.12

Activity of T6 Uvsx mutants

Key:
T6=Using T6 UvsX
T6H66T=using T6 UvsX H66T
T6M71F/S72G=using T6 UvsX M71F/S72G
T6S164V/A166S=using T6 UvsX S164V/A166S
Numbers of copies of target DNA are indicated



T6 and T6 mutants/water

Fig.13

Amplification using rb69 components

Key:
The quantity of Rb69 Uvs Y employed is indicated.
The control is template negative at a concentration of Uvs Y of 60ng/μl

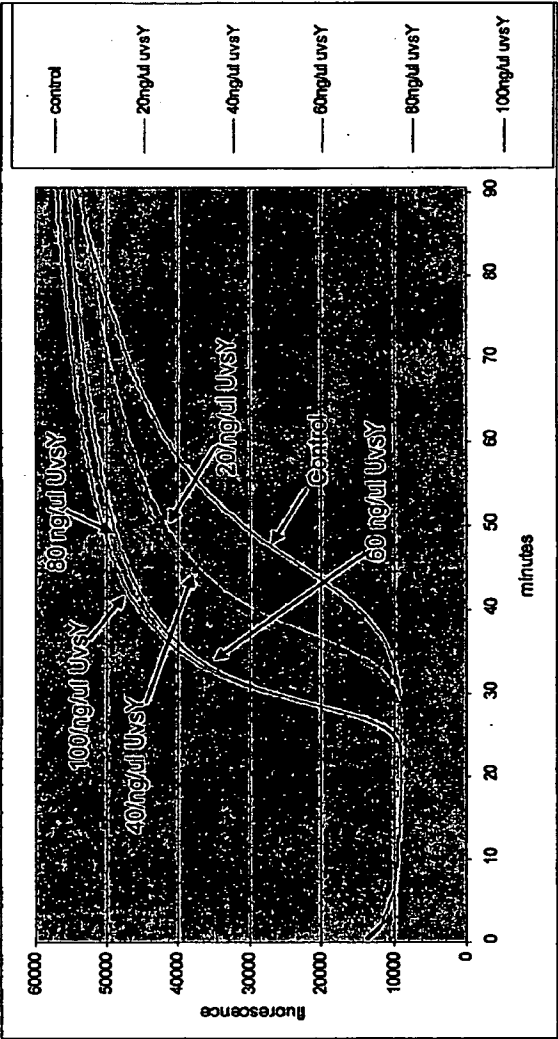


Fig.14

DNA amplification using Aeh1 components

Key:
The number of copies of target DNA are indicated

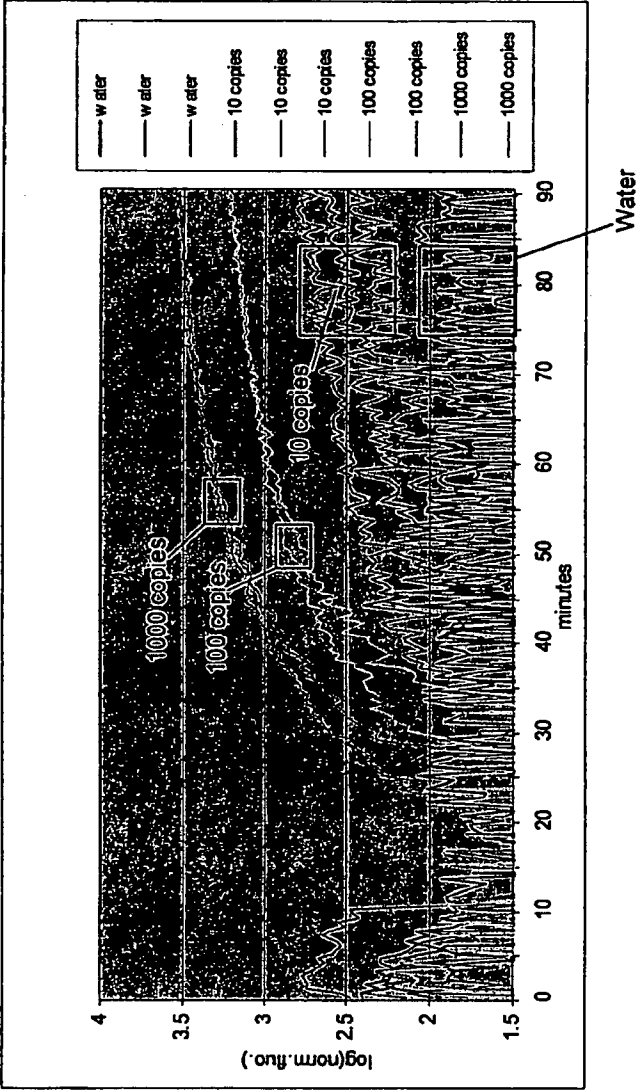


Fig.15

Amplification with Aeh1 components: salt titration

Key:
2000 copies of target DNA or water were used as indicated
Potassium acetate concentration (mM) is indicated

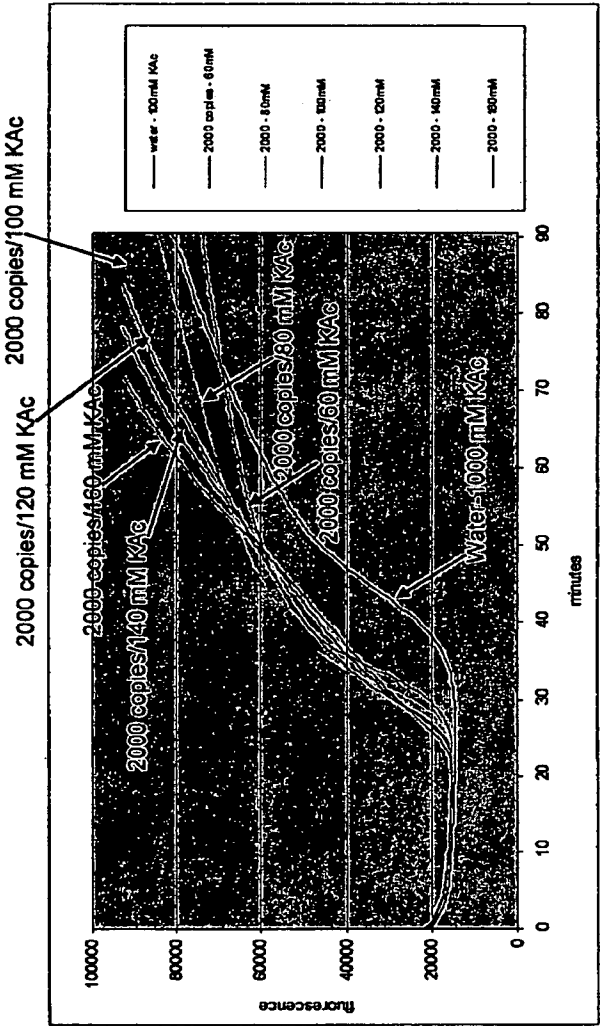


Fig.16

Comparison of the Aeh1 system to the T4 system

Key:

Aeh1 or T4 components were used as indicated.
Number of target copies is indicated.
(in this experiment there was reason to think total
copies available were a fraction of those expected.

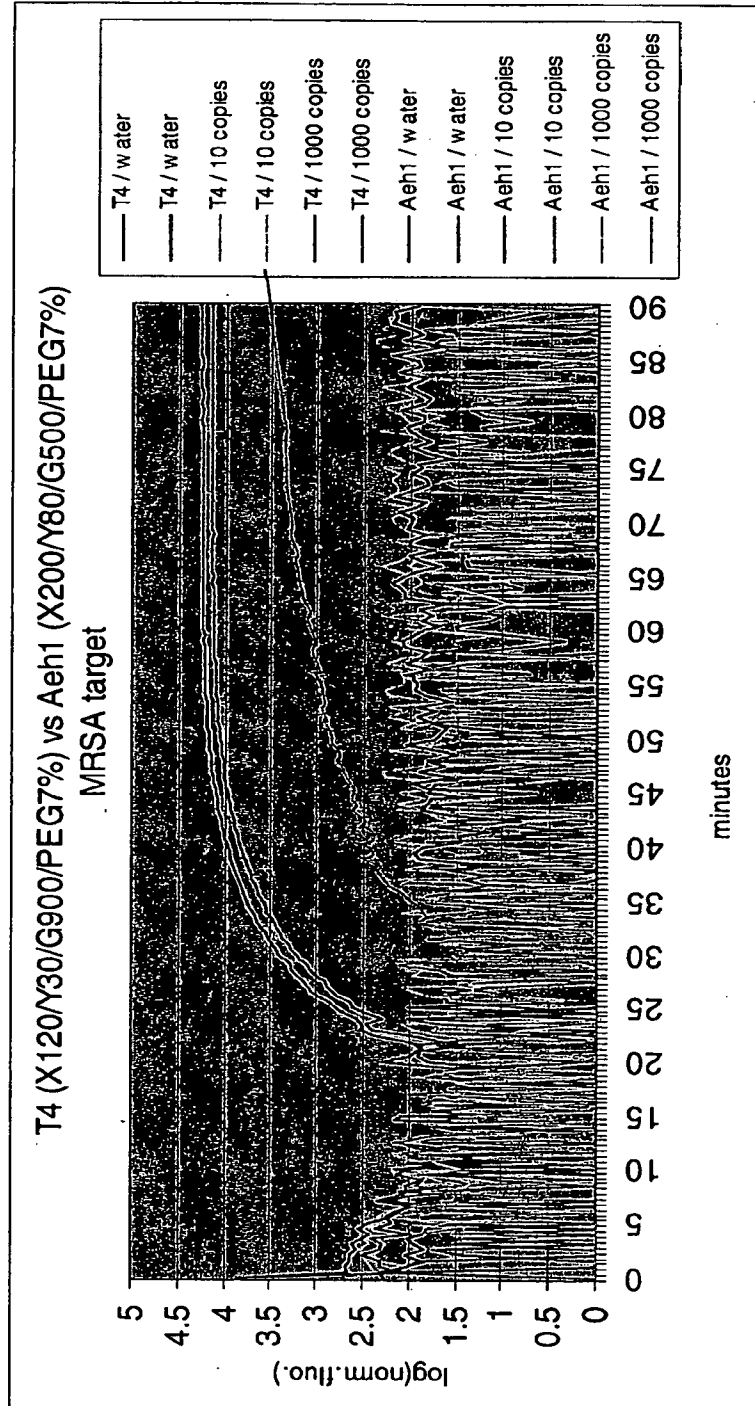


Fig.17

Experiment to test Aeh1 UvsX and UvsY: with
heterologous gp32s

Key:
The origin and amount used of gp32 is indicated.

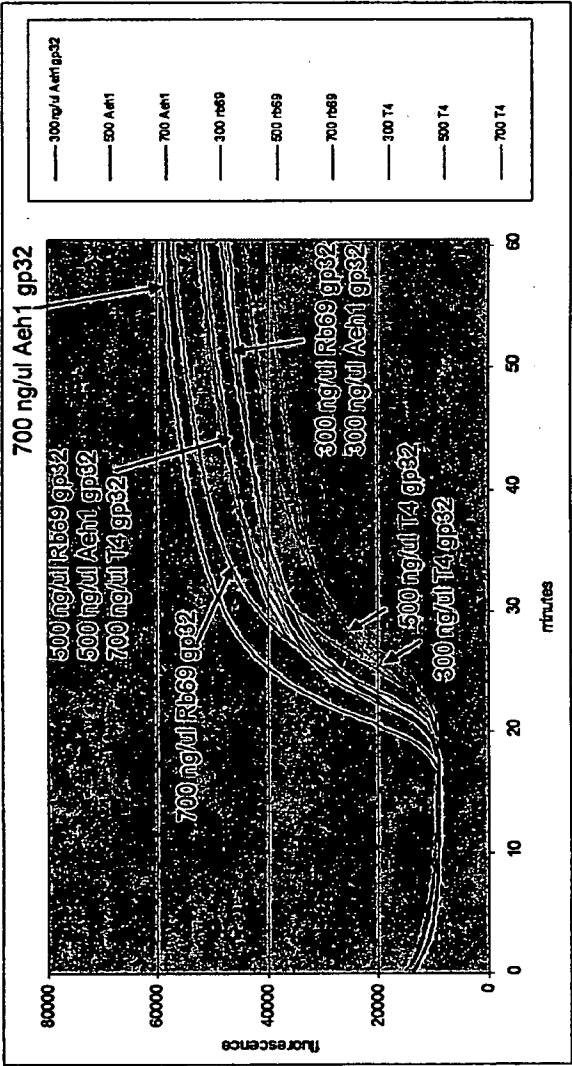
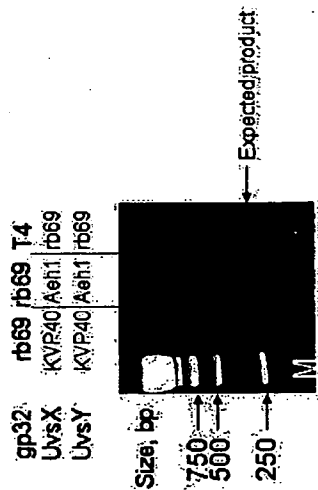


Fig.18

Heterologous amplification with rb69 gp32 and Aeh1 UvsX
and Aeh1 UvsY



Primers are Apo300 and ApoB4 which generate a ~300bp band

Target is human genomic DNA

Fig.19

Rb69 Constructs II



Activity	Clone	Loop 2's
Good ✓	WT	NHT AMEIGLYKREING GG
Low ✓	Loop133	NHT LQRIEMESKEVNT GG
None X	Loop Aeh1/Rb16	AHT YDIOENYSKVTS GG
None X	Loop Aeh1	AHT YDIOENYSKVNS GG
Pptd	Loop KV/P40	NHT YQIQETYSKVNS GG
None X	Loop Cyano	NHT YDWGSYVPTKENG GG
Proteto	Loop RecA	NOI RHIGVNEGNPETTT GG

Fig. 21

Comparison of the activities Rb69 and rb69H64S

Key:
 T4=T4 UvsX (and other components) was used.
 Rb69=Rb69 UvsX (and other components) was used.
 Rb69H64S=Rb69 H64S UvsX protein was used (with other Rb69 components).

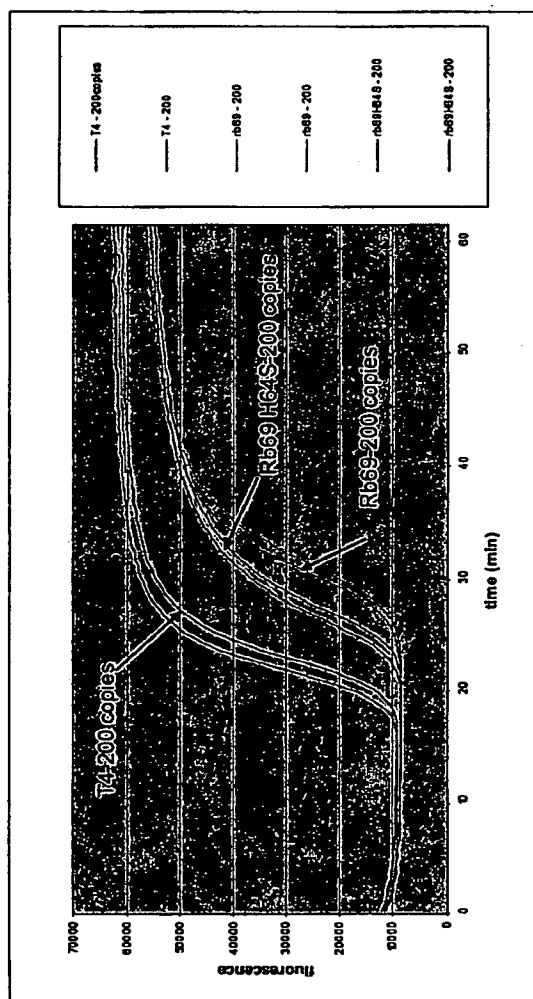


Fig.22

Comparison of the effects of Rb69 gp32 titration on amplification using Rb69 Uvsx or Rb69 UvsX H64S

Key:
Wt= wild type Rb69 UvsX was employed.
H64S= Rb69 UvsX H64S protein was employed.
The quantity of Rb69 gp32 used is indicated.

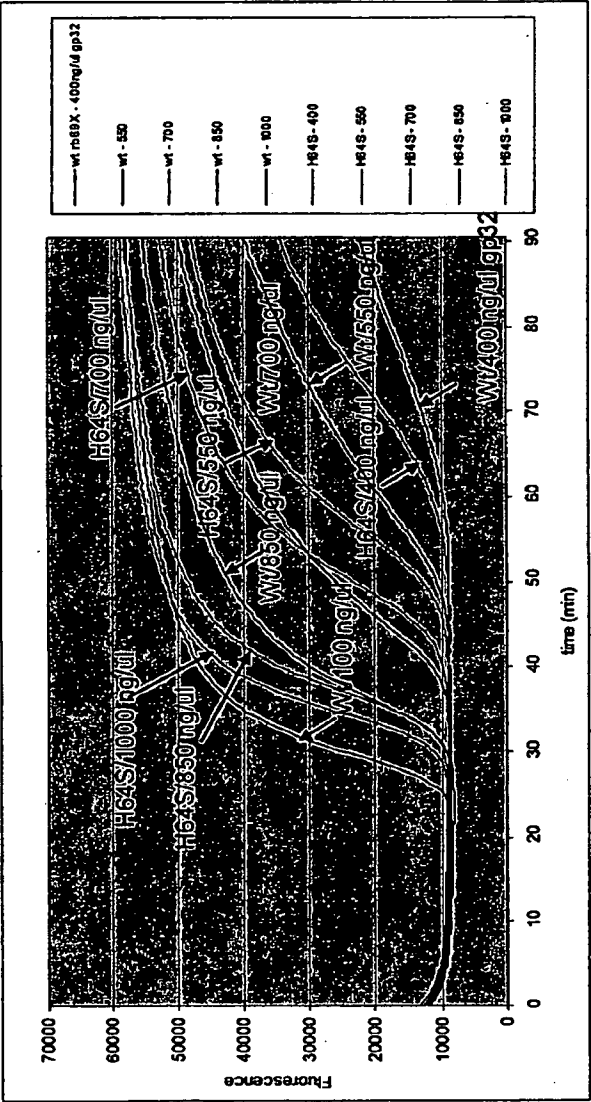


Fig.23

Comparison of Rb69Uvsx with Rb69 Uvsx H64S

Key:
Rb69= Rb69 UvsX protein was employed.
Rb69H64S= Rb69 H64S UvsX protein was employed.
The number of target copies used is indicated.

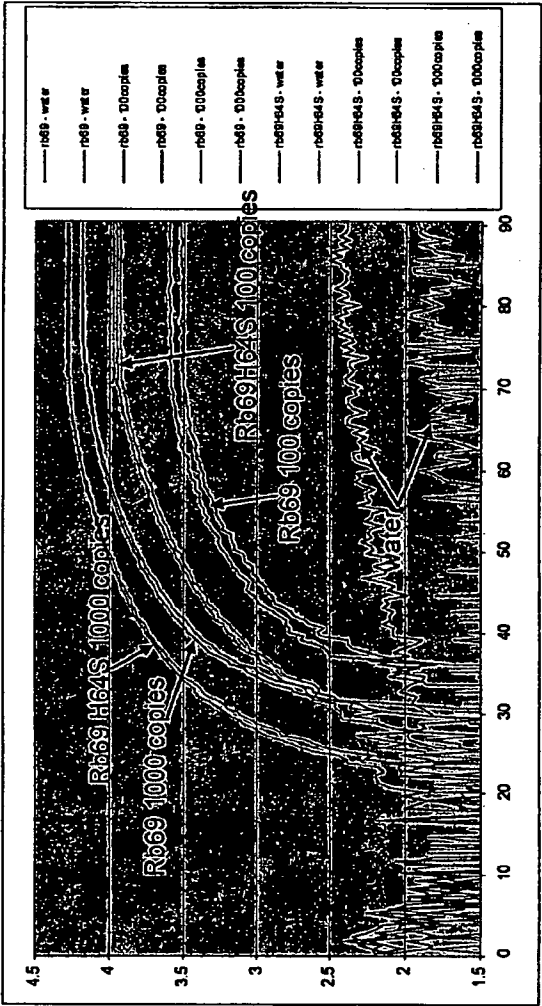


Fig.24

Rb69 Uvsx H64S protein operates over a reasonable range of Rb69gp32 concentrations

Key:

The quantity of Rb69 gp32 employed is indicated.

The number of target copies used is indicated.

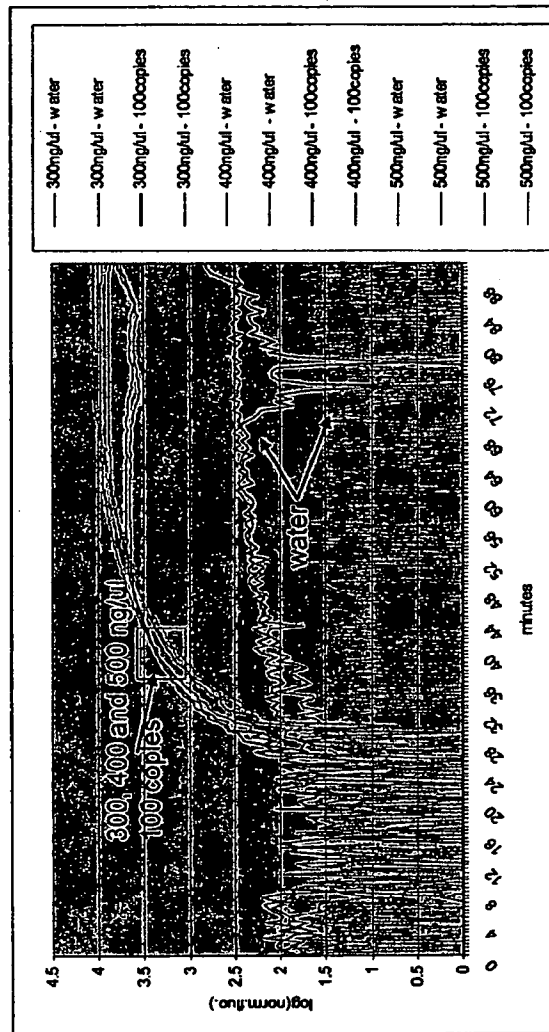


Fig.25

A titration of Rb69 Uvsx H64S

Key:
The concentration of Rb69 UvsX H64S protein used is indicated.
The target copy number used is indicated.

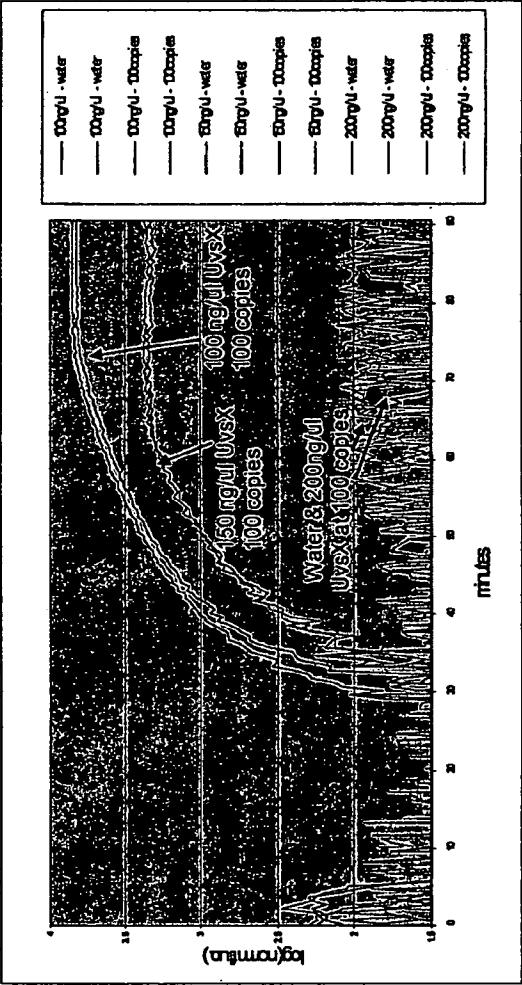


Fig.26

A titration of Rb69 Uvsx H64S

Key:
The concentration of Rb69 UvsX H64S protein used is indicated.
The target copy number used is indicated.

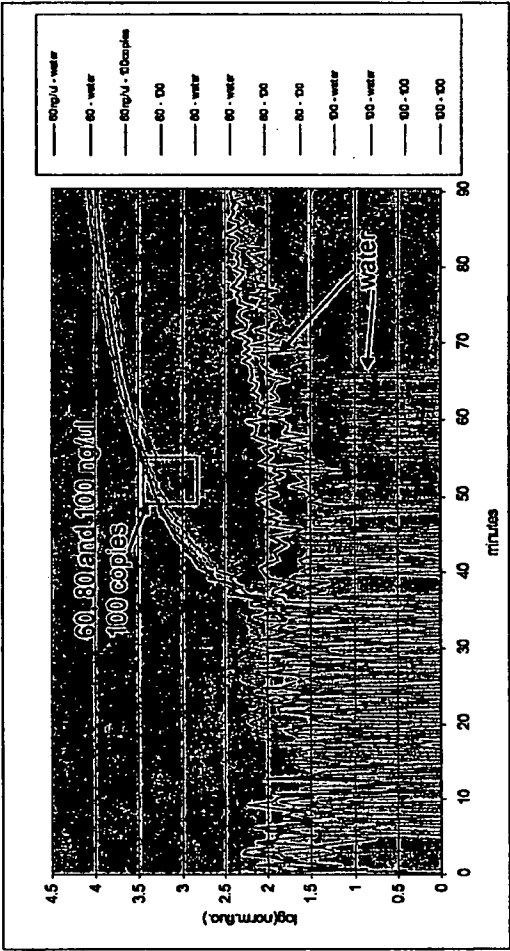


Fig.27

Effectiveness of Rb69 gp32 in reactions with T4 UvsX and UvsY.

Key:
The concentration of gp32 is indicated.
The 'type' of gp32 (T4 or Rb69) is indicated.
The target copy number is indicated.

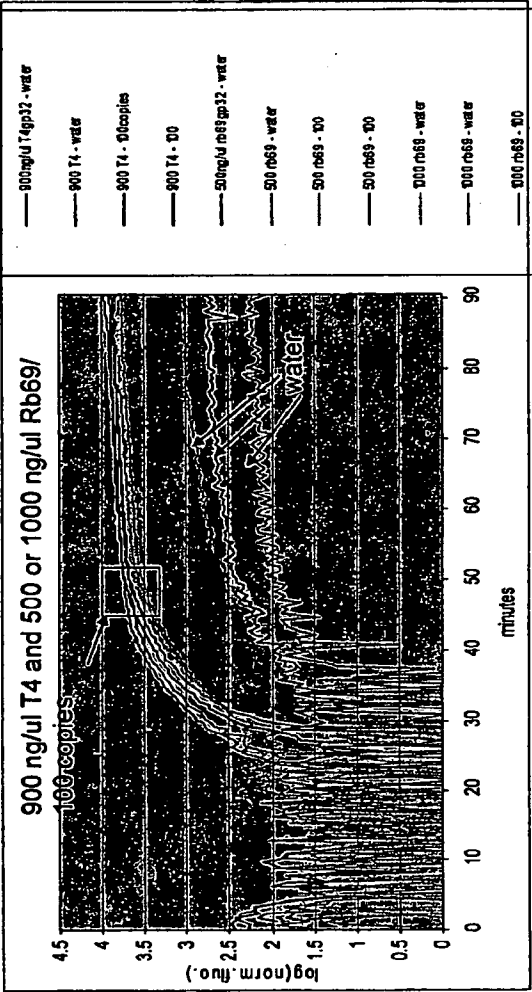


Fig.28

T4 outperforms Rb69 UvsX/UvsY system when Rb69 gp32 is used at high concentration.

Key:
The origin of the UvsX and UvsY employed is indicated (either T4 or Rb69).
The target copy number is indicated.

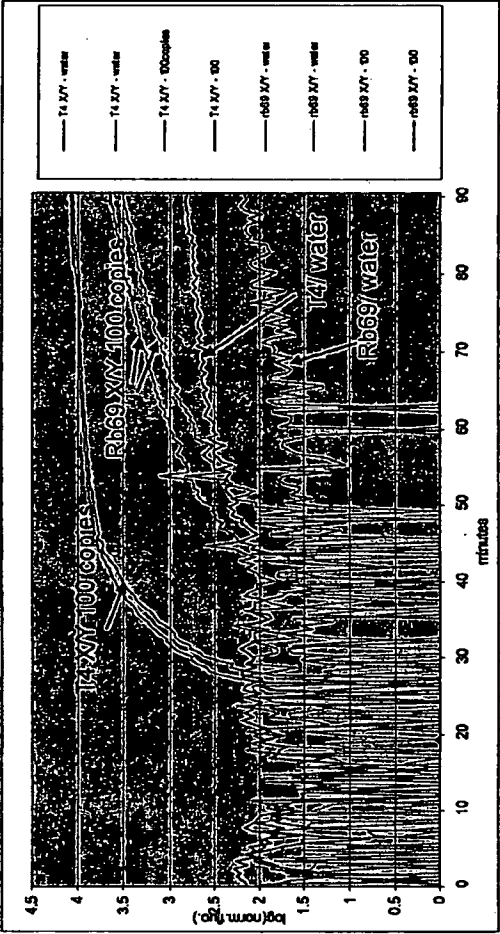


Fig.29

Poor activity of Rb69 UvsX H64T protein.

Key:
 Wt=wild type Rb69 UvsX was employed.
 H64T=Rb69 UvsX H64T was employed.
 The target copy number is indicated.

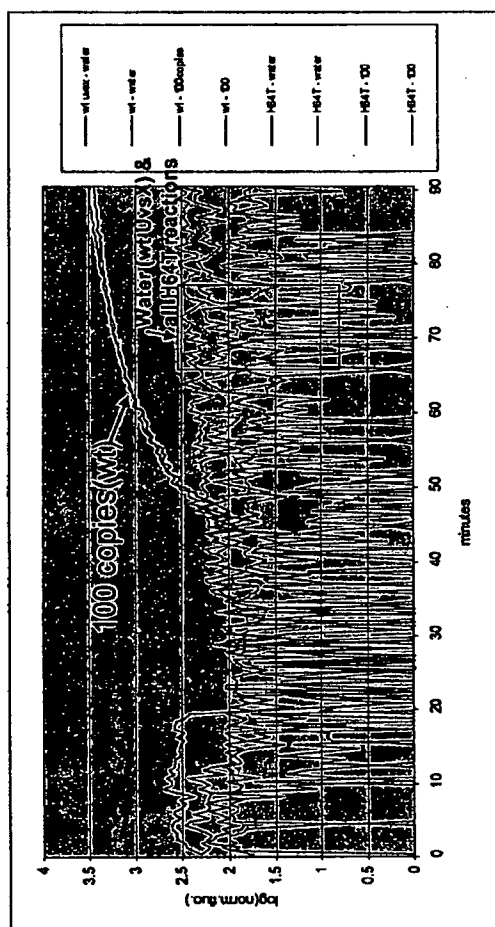


Fig.30

ATP titration using Rb69 UvsX.

Key:
The concentration of ATP employed is indicated.
The target copy number is indicated.

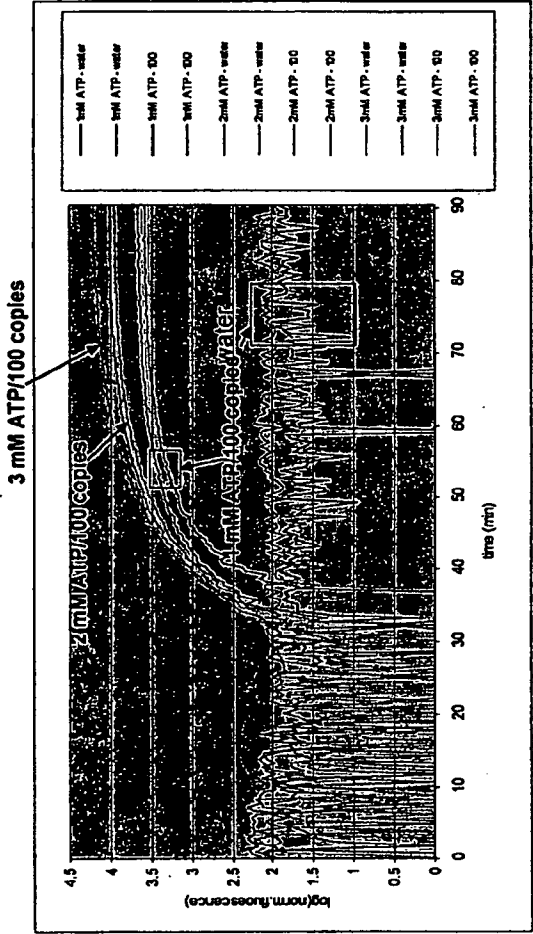


Fig.31

Suppressing effect of T4 gp32 on Rb69 UvsX and UvsY

Key:
The quantity of gp32 used is indicated.
The 'type' of gp32 employed, T4 or Rb69, is indicated.
The target copy number is indicated.

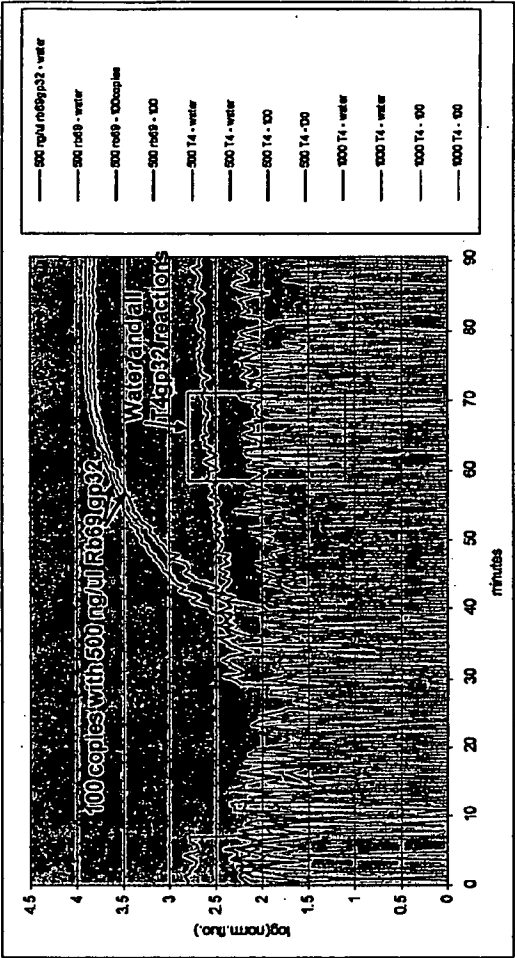


Fig. 32

Consequences of modification to the C terminus of Rb69 UvsX.

Key:

H64S=Rb69 UvsX H64S was employed.

LSD=Rb69 UvsX H64S LSD was employed.

LDELDE=Rb69 UvsX H64S 2xLDE was employed.

The target copy number is indicated.

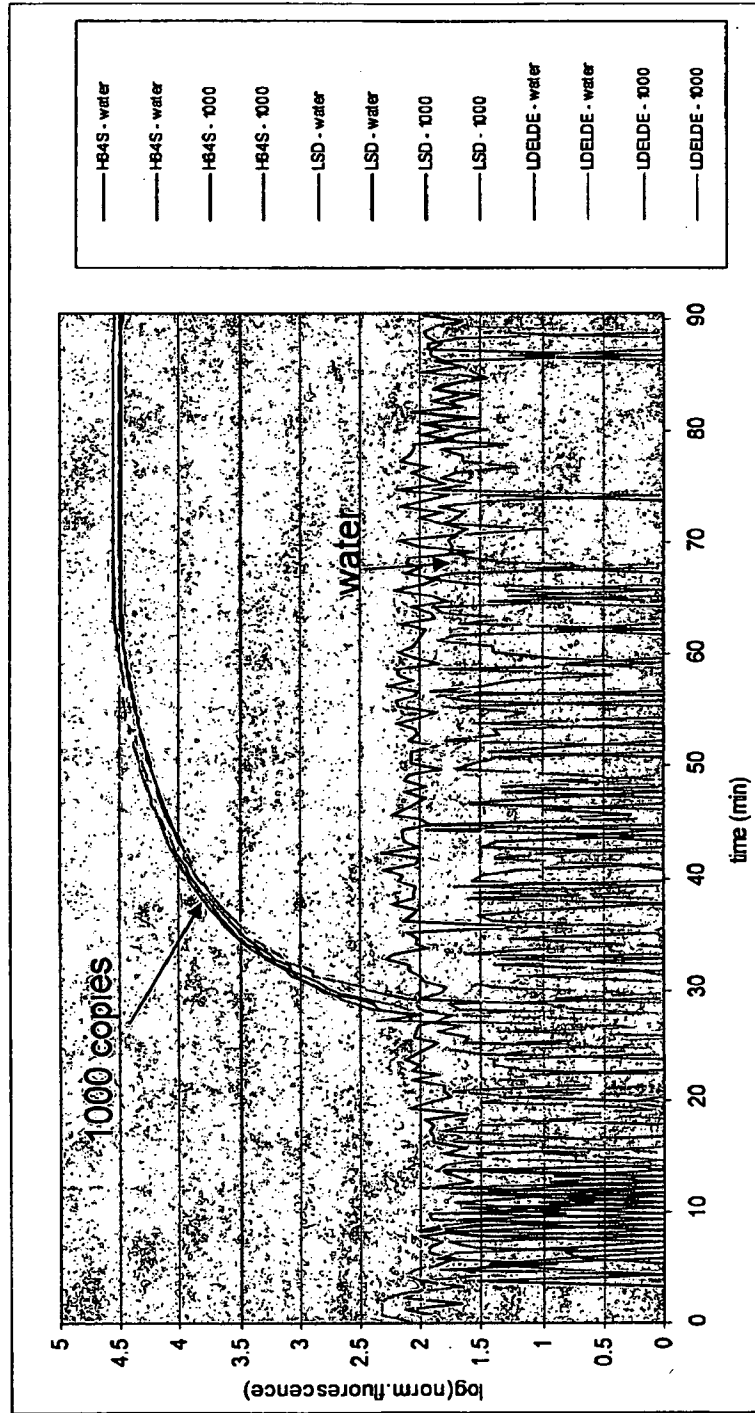


Fig.33

Titration of PEG when using Rb69 UvsX H64S 2xLDE.

Key:
The concentration of PEG 35,000 (Fluka) is indicated.
The target copy number is indicated.

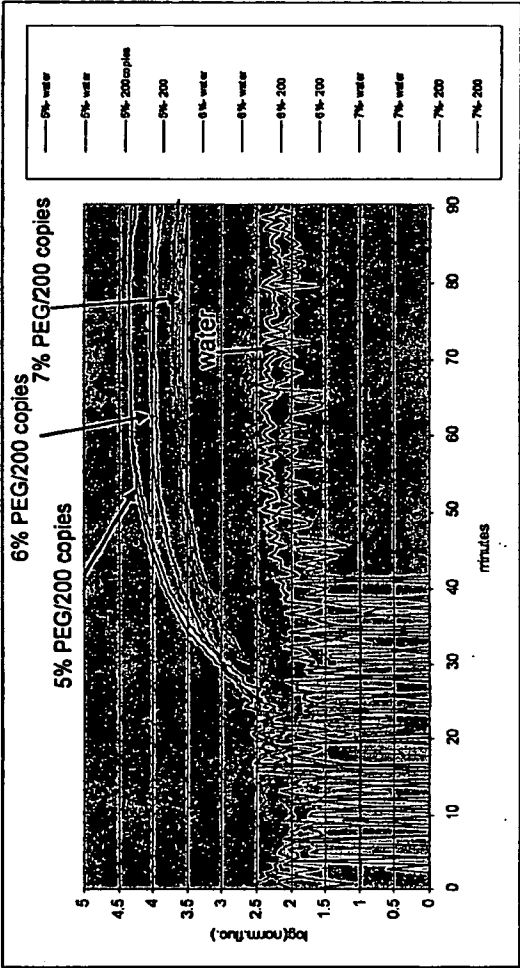


Fig.35

Hybrid UvsY Proteins

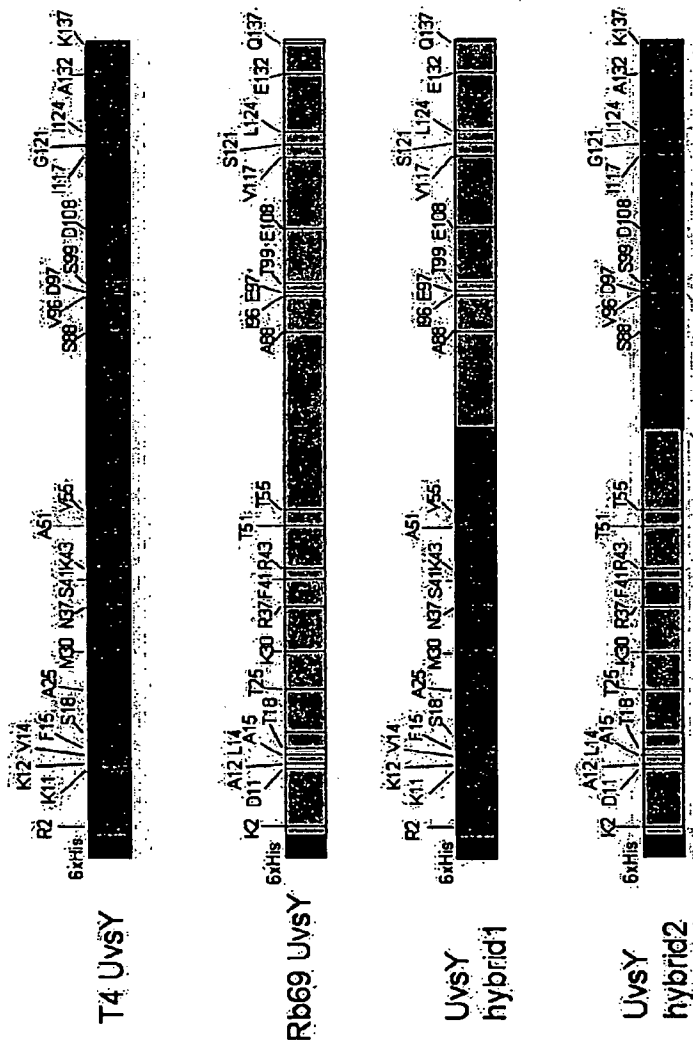


Fig.36

Activity of UvsY hybrids with T4 UvsX and T4 gp32.

Key:
T4= T4 UvsY was employed.
T4-rb=UvsY hybrid 1 was employed.
Rb-T4=UvsY hybrid 2 was employed.
Rb= Rb69 UvsY was employed.
Target copy numbers are indicated.

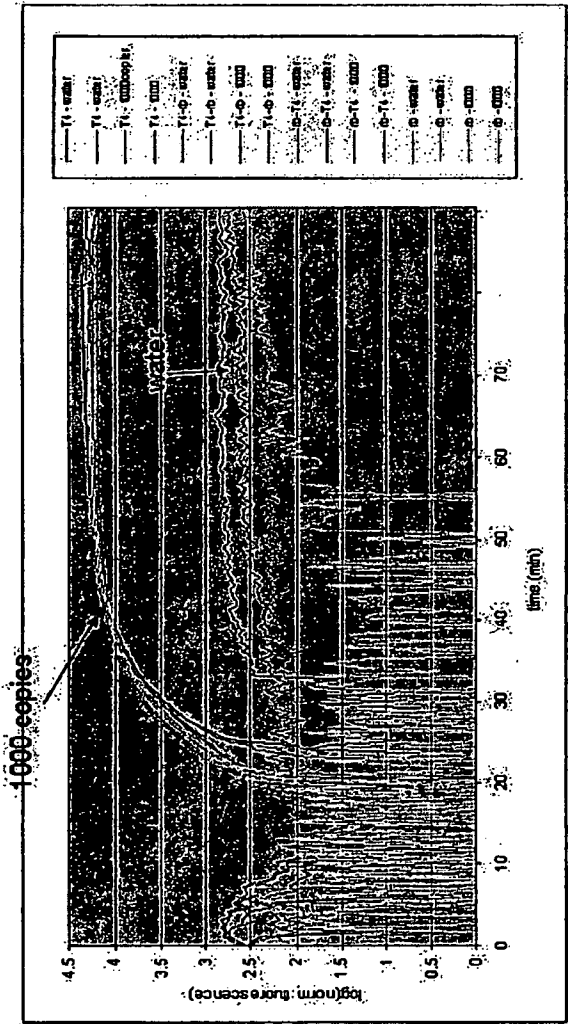


Fig.37

Activity of UvsY hybrids with Rb69 UvsX and Rb69 gp32.

Key:

T4= T4 UvsY was employed.

T4-rb= UvsY hybrid 1 was employed.

Rb-T4= UvsY hybrid 2 was employed.

Rb= Rb69 UvsY was employed.

Target copy numbers are indicated.

T4-rb and Rb-T4

T4-1000 copies 1000 copies

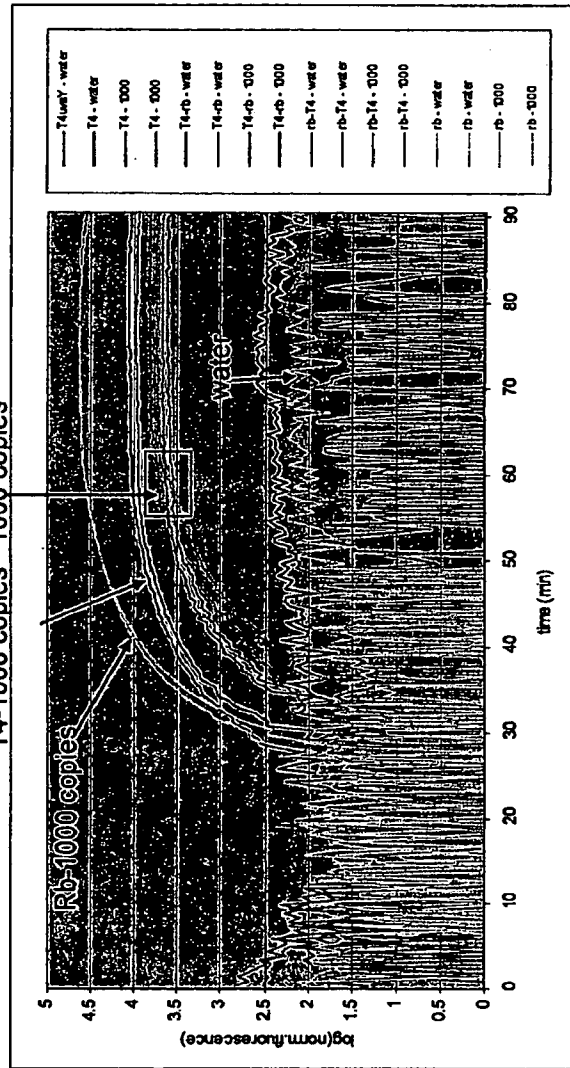


Fig.38

No activity for Rb69 Uvsx H64S T6-1 2xLDE.

Key:
 LDE=Rb69 UvsX H64S 2xLDE protein was employed.
 Loop2=Rb69 UvsX H64S T6-1 2xLDE protein was employed.
 Target copy numbers are indicated.

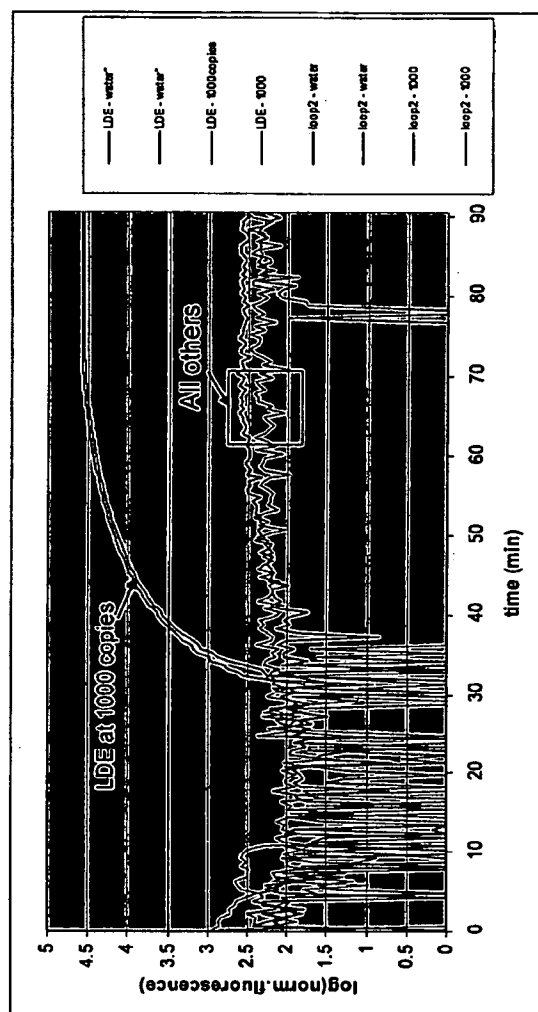


Fig.39

Titration of Rb69 gp32 in the presence of Rb69 Uvsx H64S 2xLDE.

Key:
The quantity of Rb69 gp32 employed is indicated.
The target copy number is indicated.

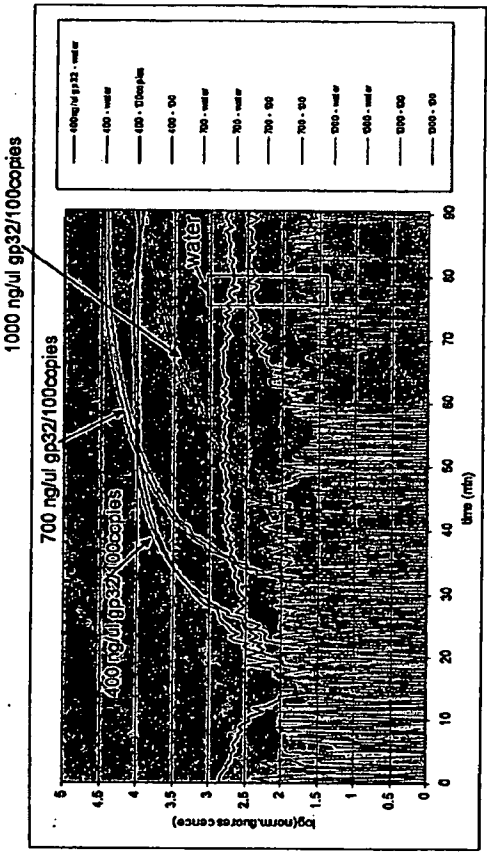


Fig. 40

No activity for Rb69 UvsX H64S F69M G70S T6-1 2xLDE

Key:
LDE=Rb69 UvsX H64S 2xLDE was employed.
Mutant=Rb69 UvsX H64S F69M G70S T6-1 2xLDE was employed.

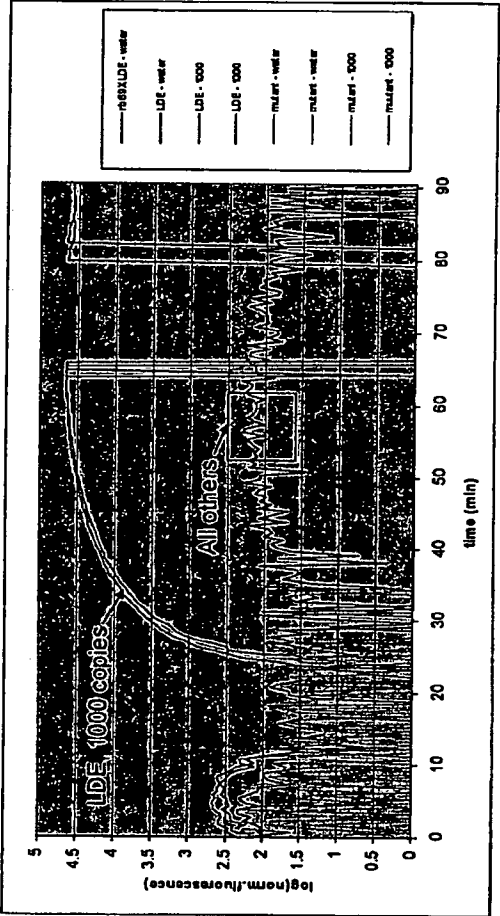


Fig. 41

Strong activity of Rb69 H64S T67S/L68N T4 2xLDE.

Key:
Wt=wild type Rb69 UvsX was employed.
LDE=Rb69 UvsX H64S 2xLDE was employed.
T4/T4= Rb69 H64S T67S/L68N T4 2xLDE was employed.
Target copy numbers are indicated.

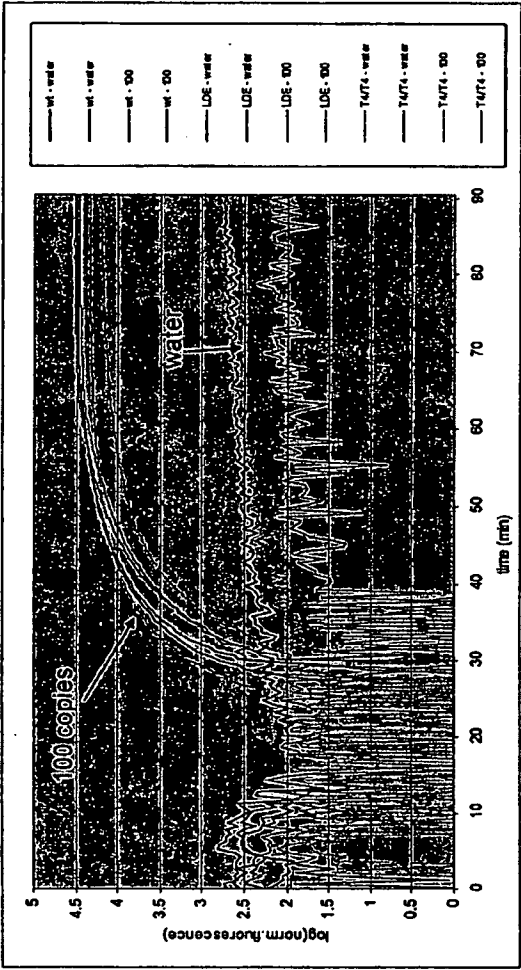


Fig. 42

Rb69 UvsX H64S T67S/L68N T4 2xLDE protein is relatively resistant to up-titration of Rb69 gp32.

Key:
Wt= wild type Rb69 UvsX was employed.
Mut= Rb69 UvsX H64S T67S L68N T4 2xLDE protein was employed.
The quantity of Rb69 gp32 (400ng/ μ l or 800ng/ μ l) is indicated.
The target copy number (0 or 100) is indicated.

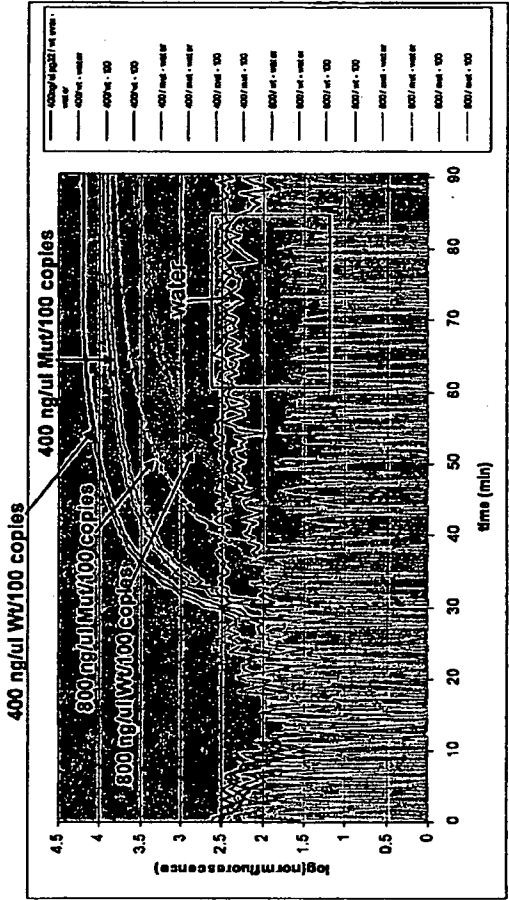


Fig. 43

Rb69 UvsX H64S T67S/L68N T4 2xLDE protein can function with T4 gp32.

Key:

T4=T4 UvsX, UvsY and gp32 were employed.

Rb69=Rb69 UvsX and UvsY were employed with T4 gp32.

T4like= Rb69 UvsX H64S T67S/L68N T4 2xLDE and Rb69 UvsY were employed with T4 gp32.

Target copy numbers are indicated.

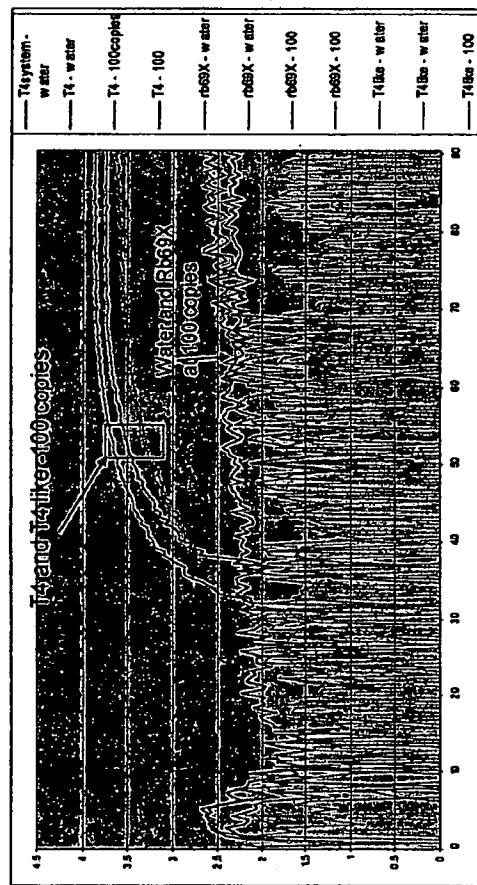


Fig. 44

Rb69 UvsX chimeras containing DNA-binding loops from phage 133 work weakly, while cyanophage and Aeh1 loops are non-functional.

Key:

- Wt=wild type Rb69 UvsX was employed.
- Act133=Rb69loop133 was employed.
- CyPh=Rb69loopcyano was employed.
- Aeh=Rb69 loopAeh1 was employed.
- Target copy numbers are indicated.

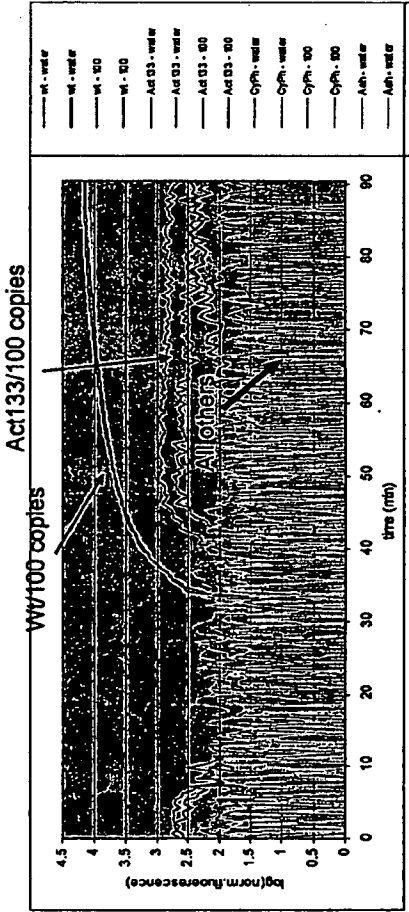


Fig. 45

Rb69 UvsX H64S T6 2xLDE is active unlike the equivalent lacking the final G to T substitution of the DNA-binding loop2.

Key:
Wt=wild type Rb69 UvsX was employed.
rb69xLDE or LDE=Rb69 H84S 2xLDE was employed.
T6loop2+Thr or Thr = Rb69 UvsX H64S T6 2xLDE was employed.
Rb16loop2 or rb16=Rb69 UvsX Loop Aeh1/Rb16 was employed.

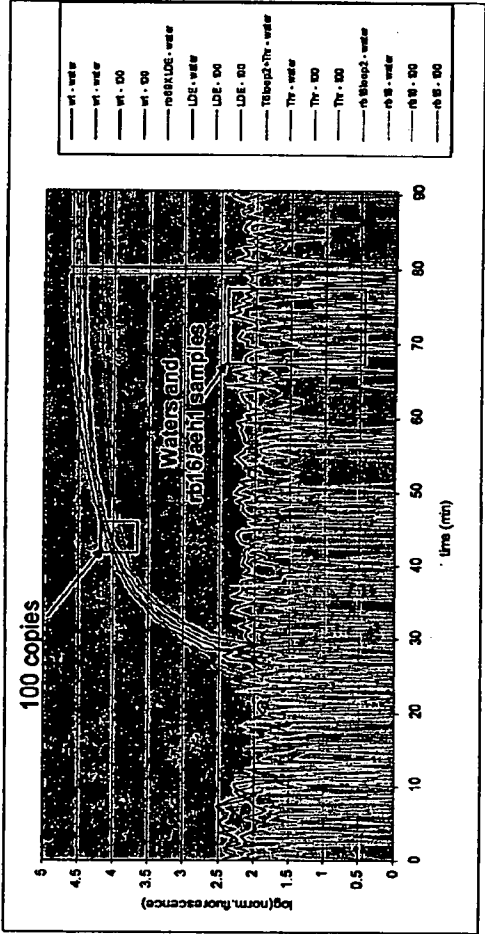


Fig.46

Manganese ions can support RPA reactions

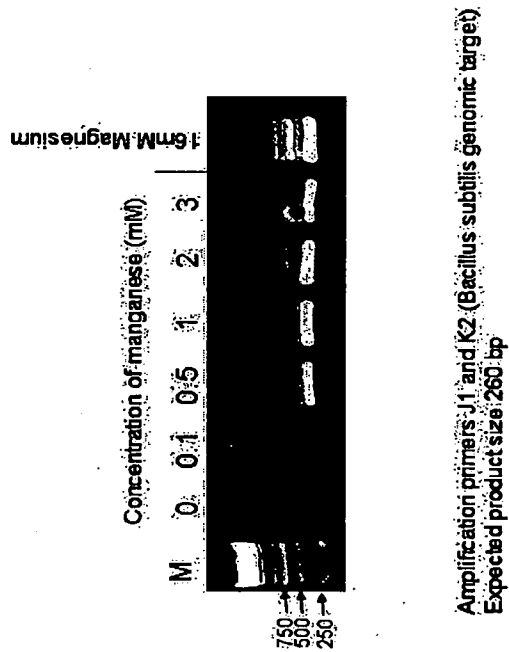


Figure 47

S. Aureus Pol I works well in RPA

Key:
The species of polymerase employed is indicated.
The target copy number is indicated.

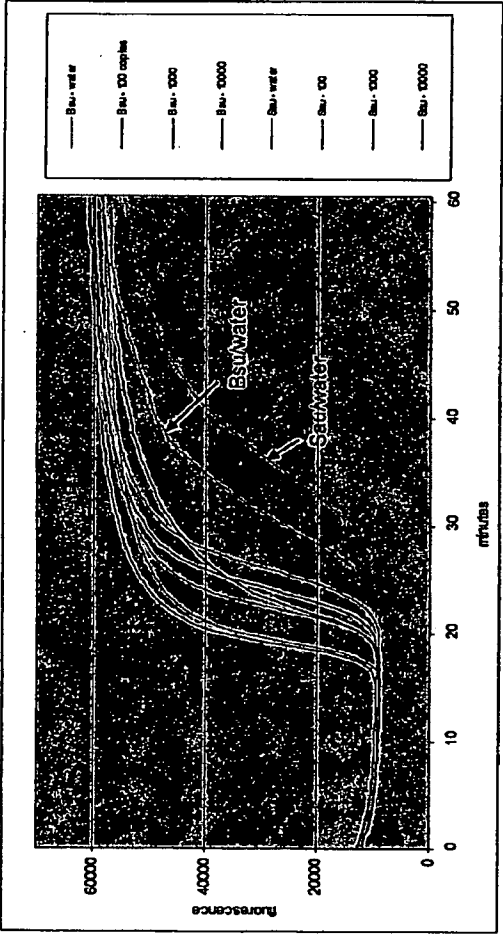


Figure 48

Heparin delays the onset of noise detection in water controls

Key:

The presence or absence of heparin is indicated.

No targets were included in these reactions.

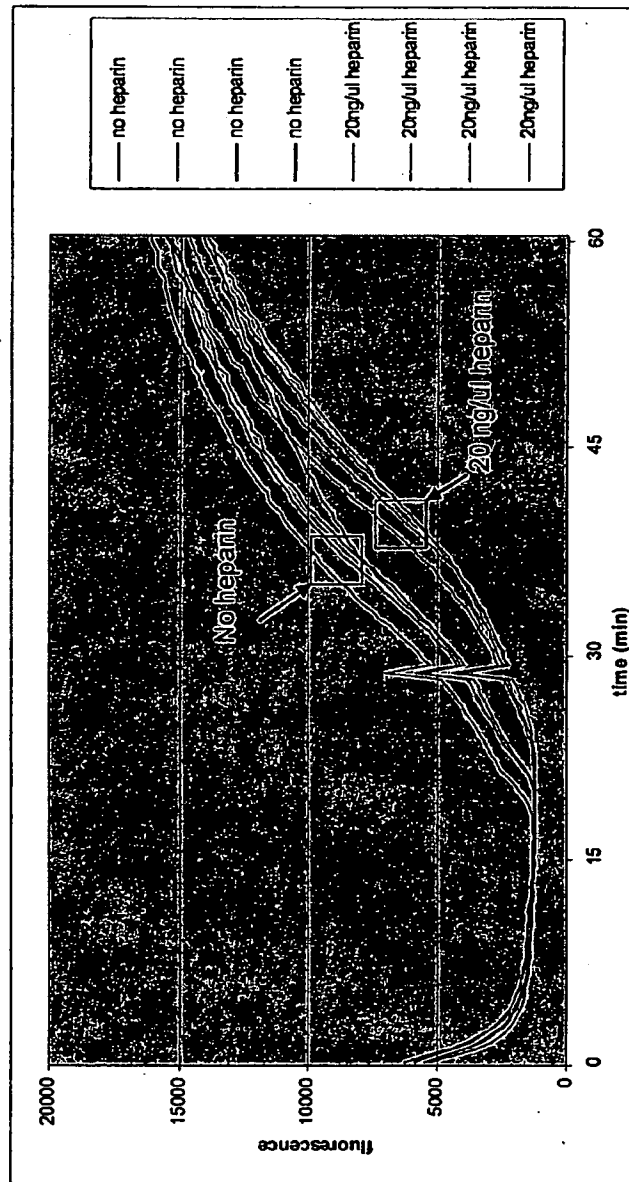


Figure 49

Heparin improves the resolution of target numbers at low copy number

Key:
The target copy numbers are indicated.
The presence or absence of heparin is indicated.

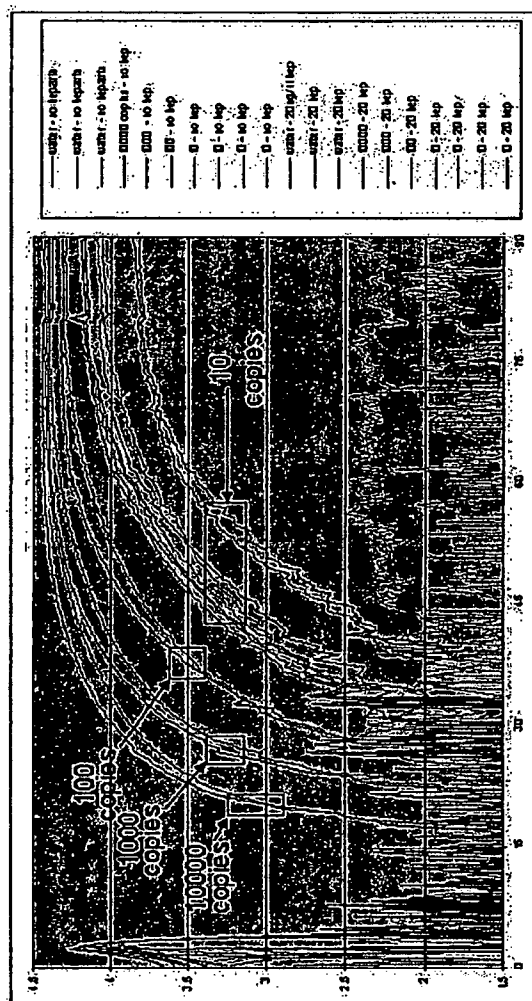


Figure 50

Blocked primers can be used in RPA

Key:
The species of primers used is indicated.
The use of exo III or Nfo is indicated.
The target copy numbers used are indicated.

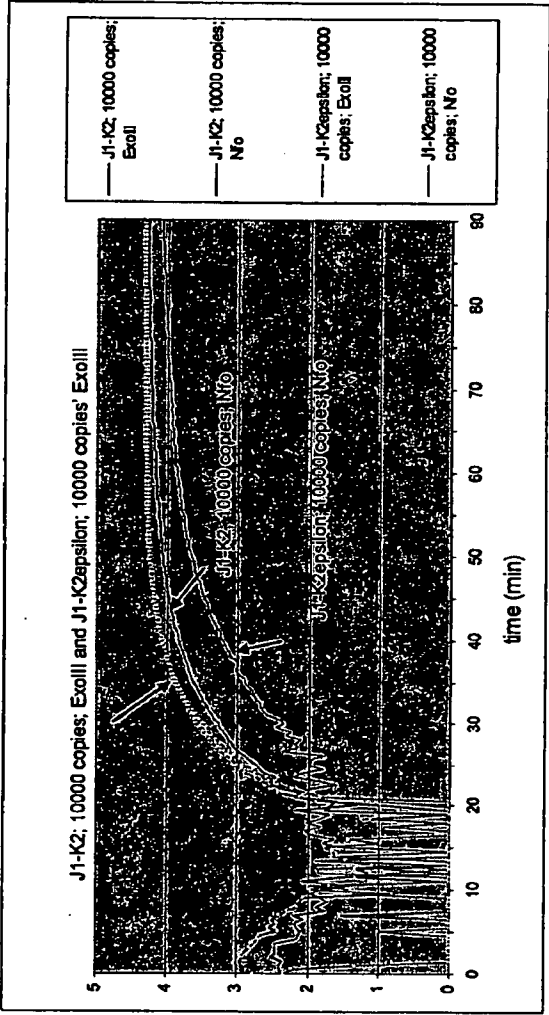


Figure 51

DNA amplification in the absence of uvsY

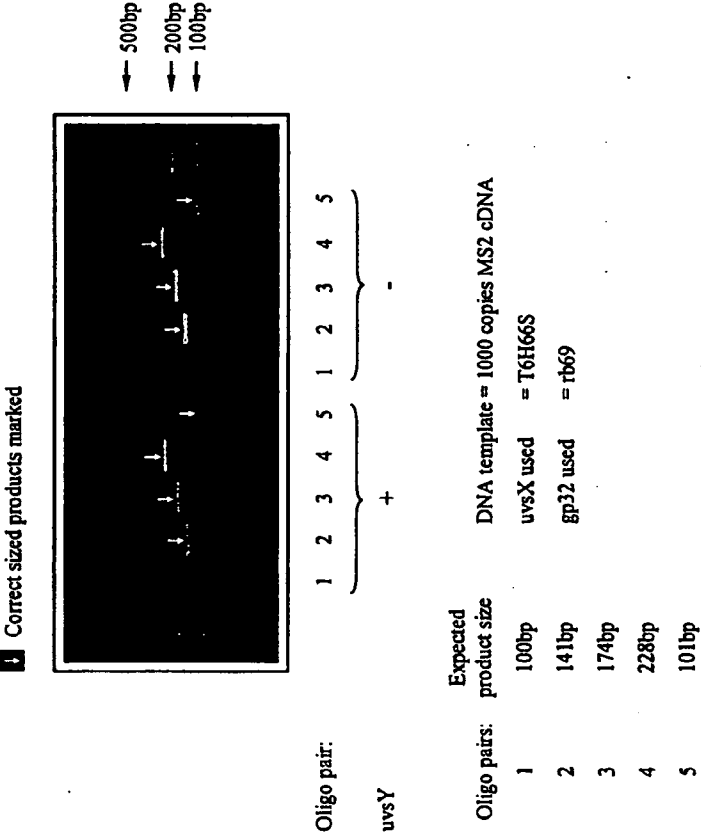


Figure 53

uvrY presence and absence test using a short DNA template

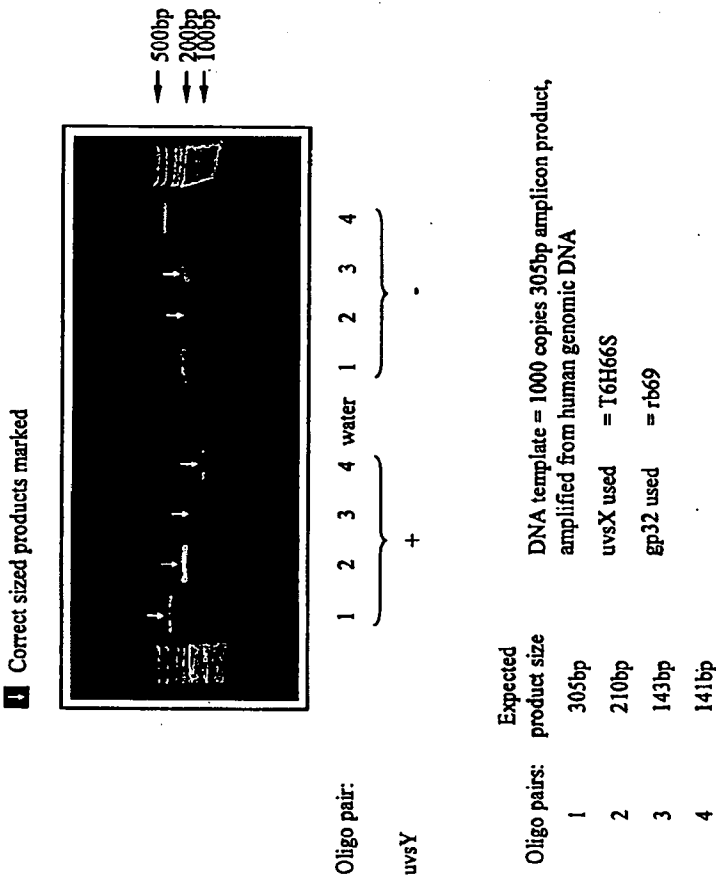


Figure 54

DNA amplification in the absence of uvsY occurs when using genomic DNA

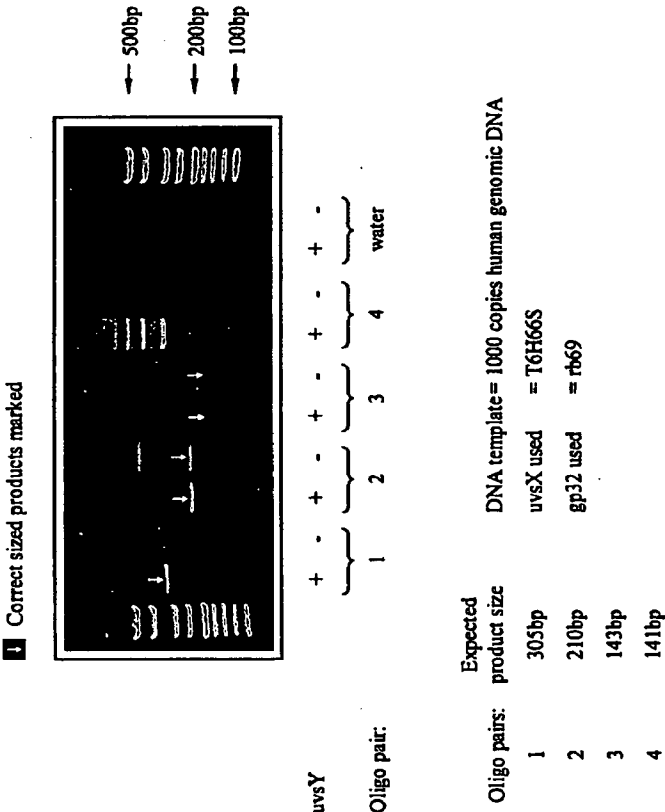


Figure 55

No DNA amplification in the absence of uvsY following the removal of PEG

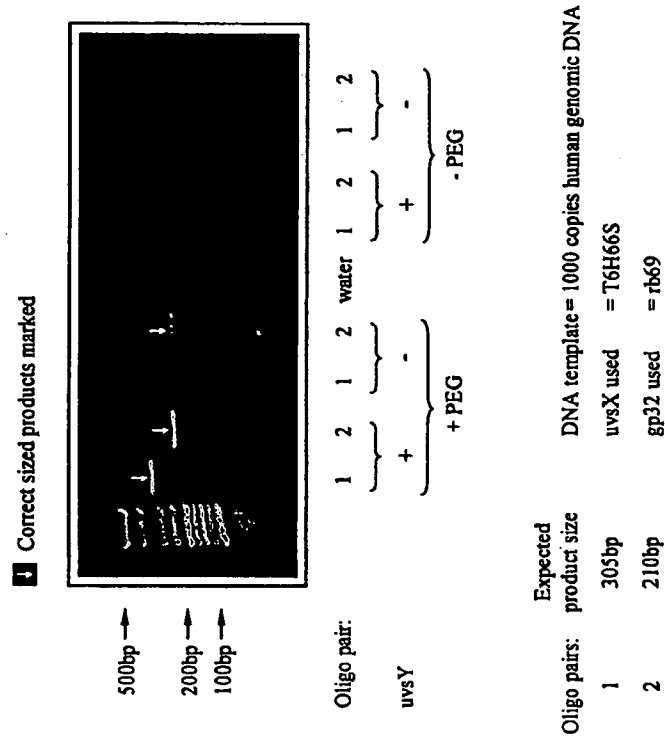


Figure 56

DNA amplification in the absence of uvsY using T4 gp32

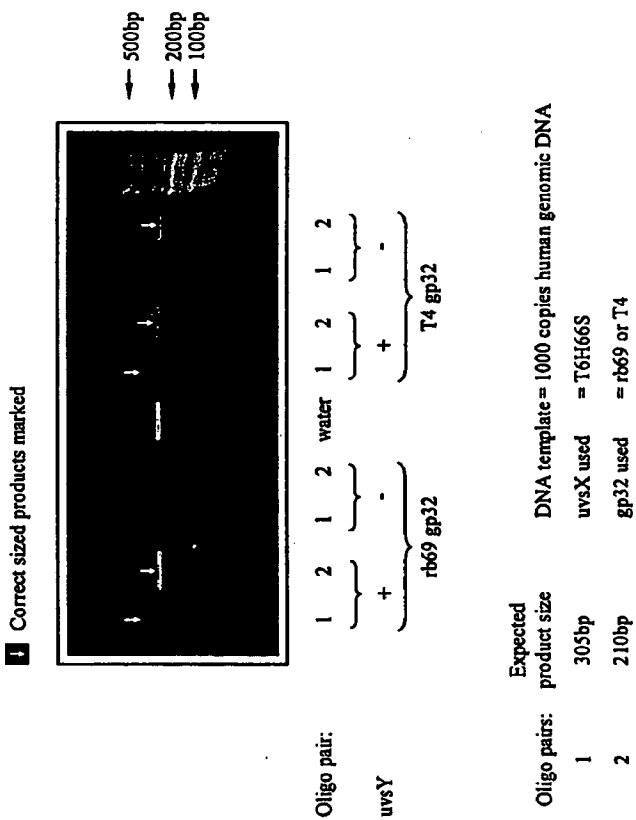


Figure 57

Poor DNA amplification in the absence of uvsY using AehI gp32

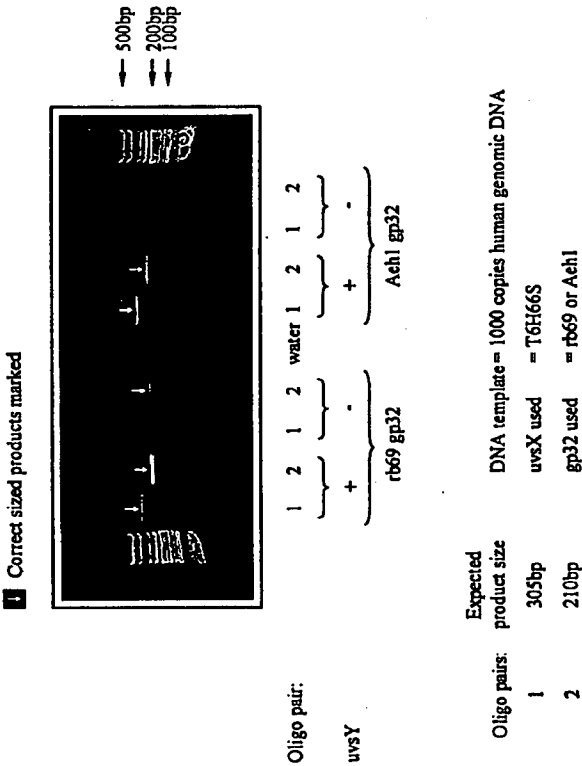


Figure 58

No DNA amplification in the absence of uvsY using T4 uvsX with Rb69 gp32

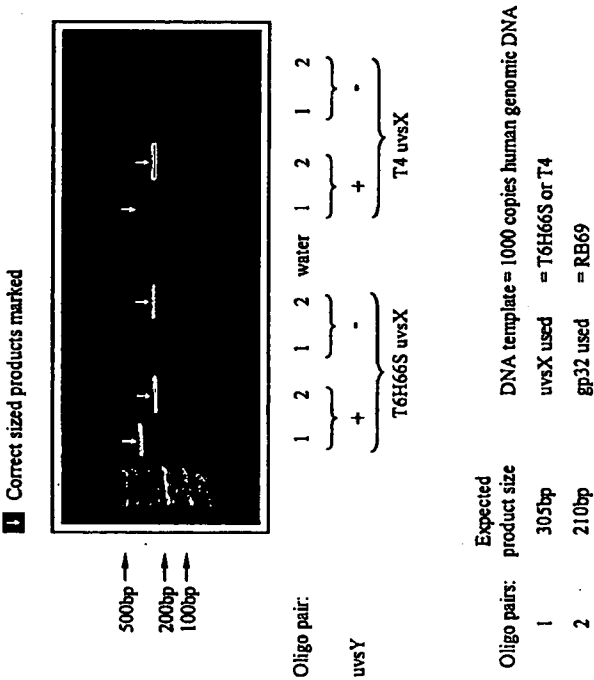


Figure 59

No DNA amplification in the absence of uvsY using T4 uvsX with T4 gp32

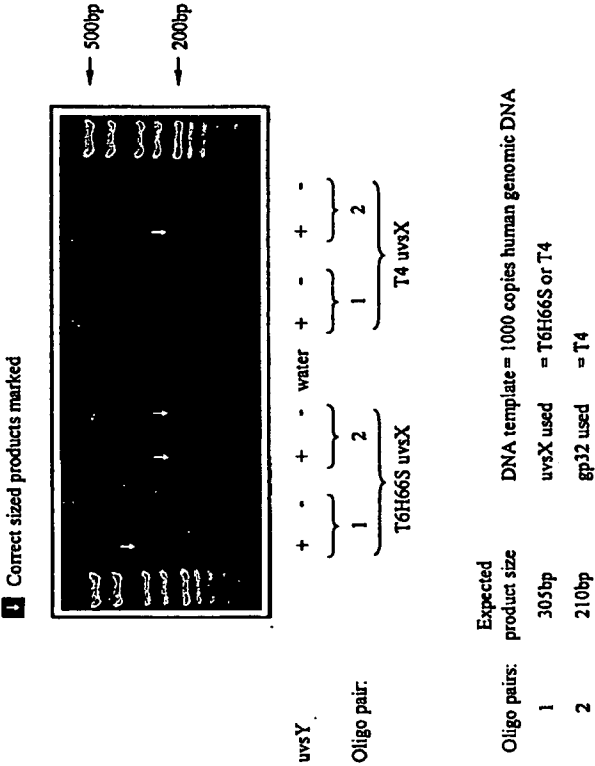
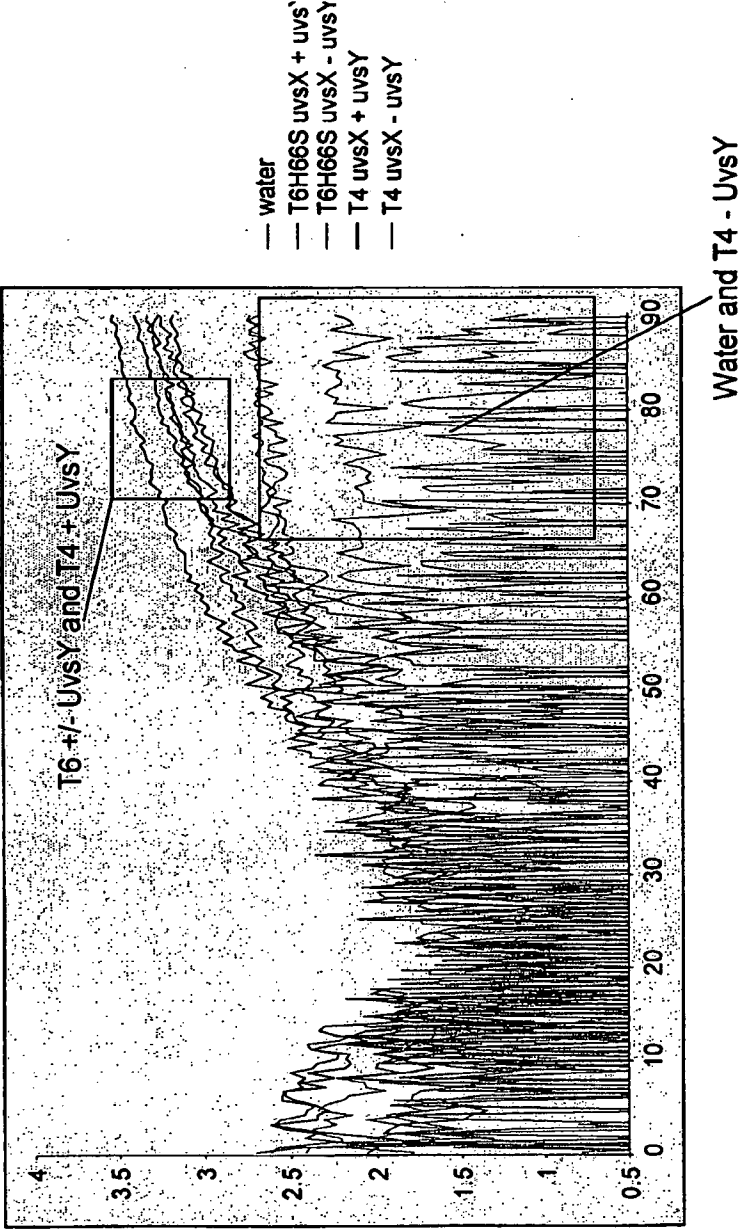


Figure 60



DNA template = 200 copies Bacillus Subtilis DNA

uvsX used = T6H66S or T4

gp32 used = rb69

Figure 61

DNA amplification in the absence of uvsY using T6 uvsX

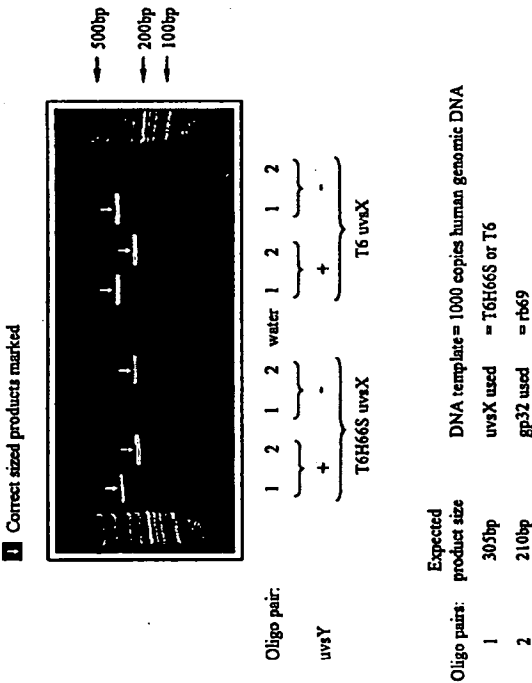


Figure 62

No DNA amplification in the absence of uvsY using rb69 uvsX

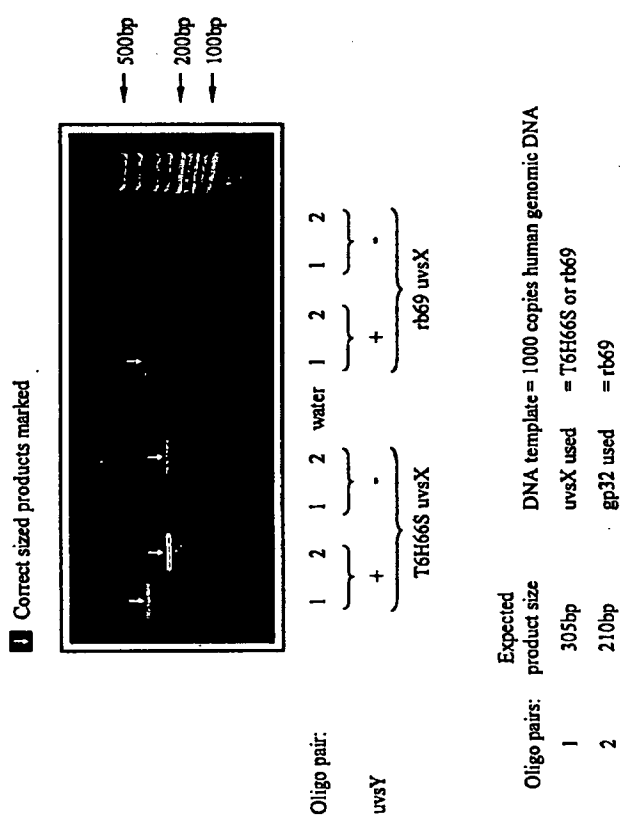


Figure 63

No DNA amplification in the absence of uvsY using Aeh1 uvsX

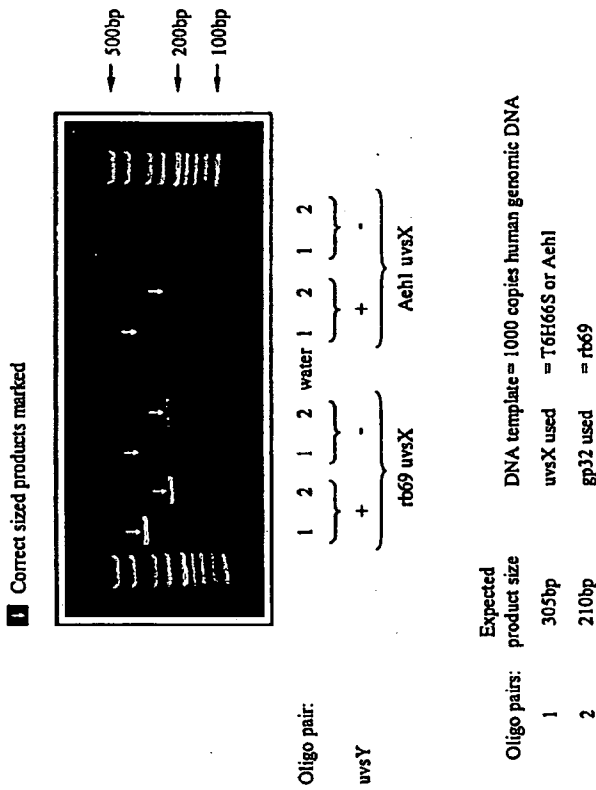


Figure 64

No DNA amplification in the absence of uvsY using Rb69 T6loop2H64S
2xLDE uvsX

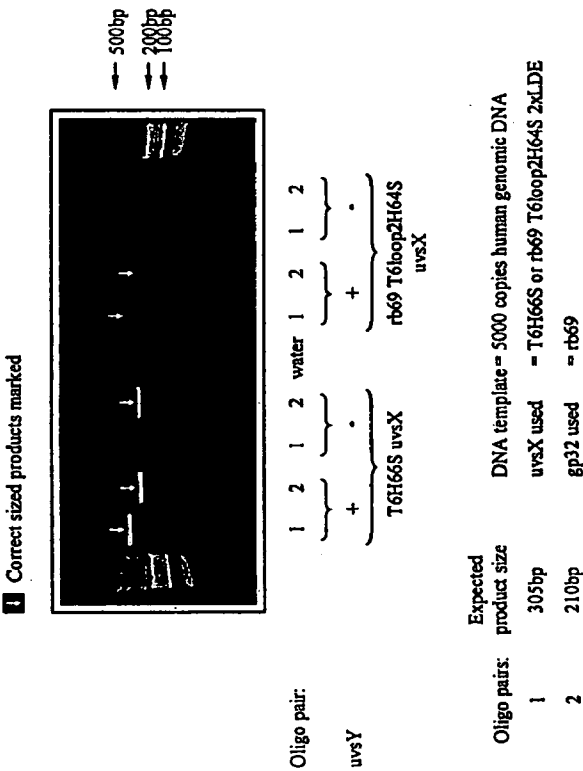


Figure 65

Reaction setup (in 1xNEB4 buffer):

- present at start: 100nM probe (SA Tamra2: Tamra/THF/BHQ2)
- present at start: indicated amounts of rb69 gp32
- present at start: Nfo (prep 17/1; final dilution 1in300)

Conclusion:

dose response for probe protection by gp32

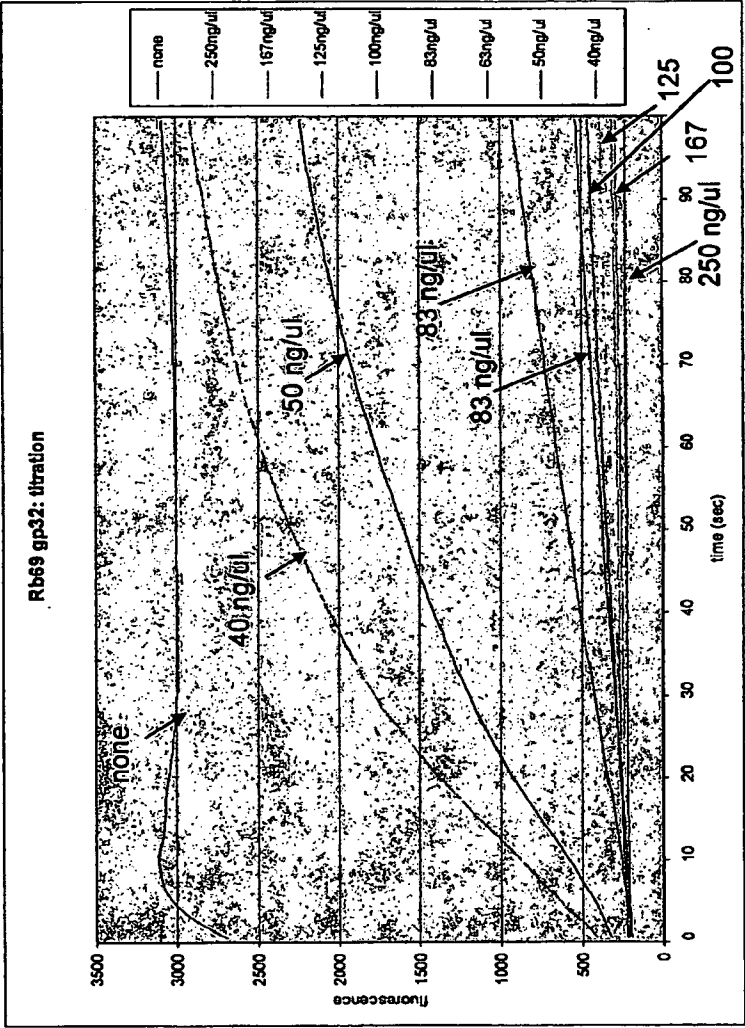


Figure 66

Gp32 homologs: ssDNA binding versus dsDNA binding

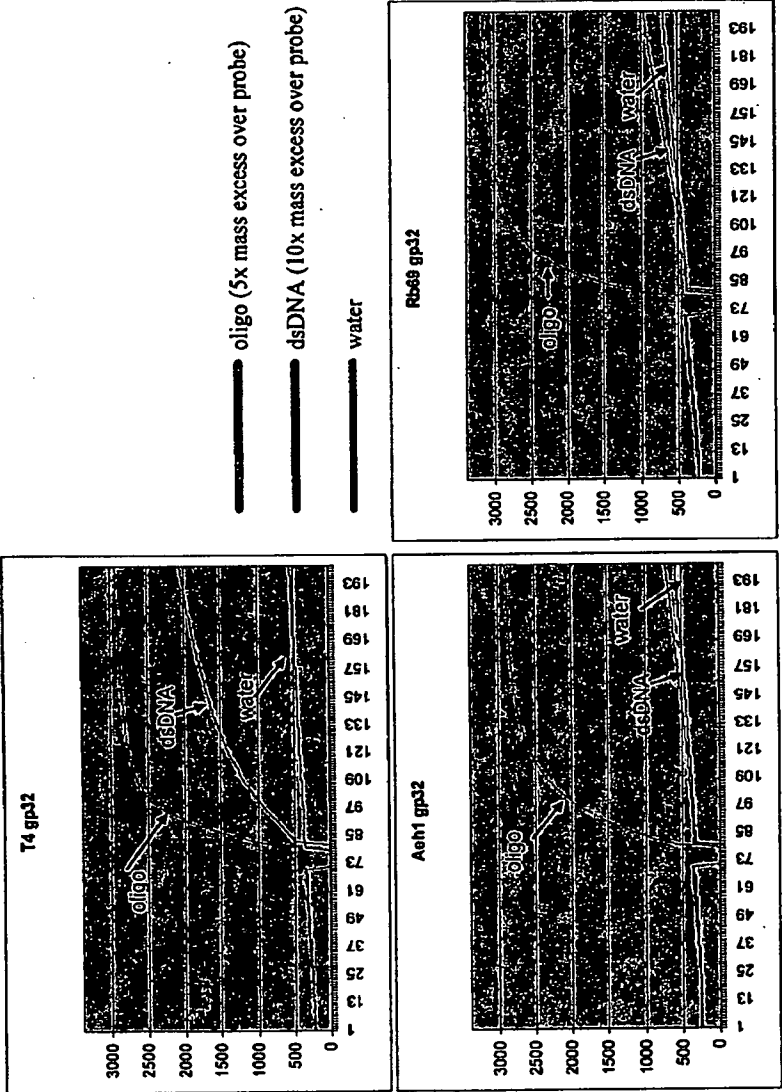


Figure 67

Gp32 homologs: temperature effects

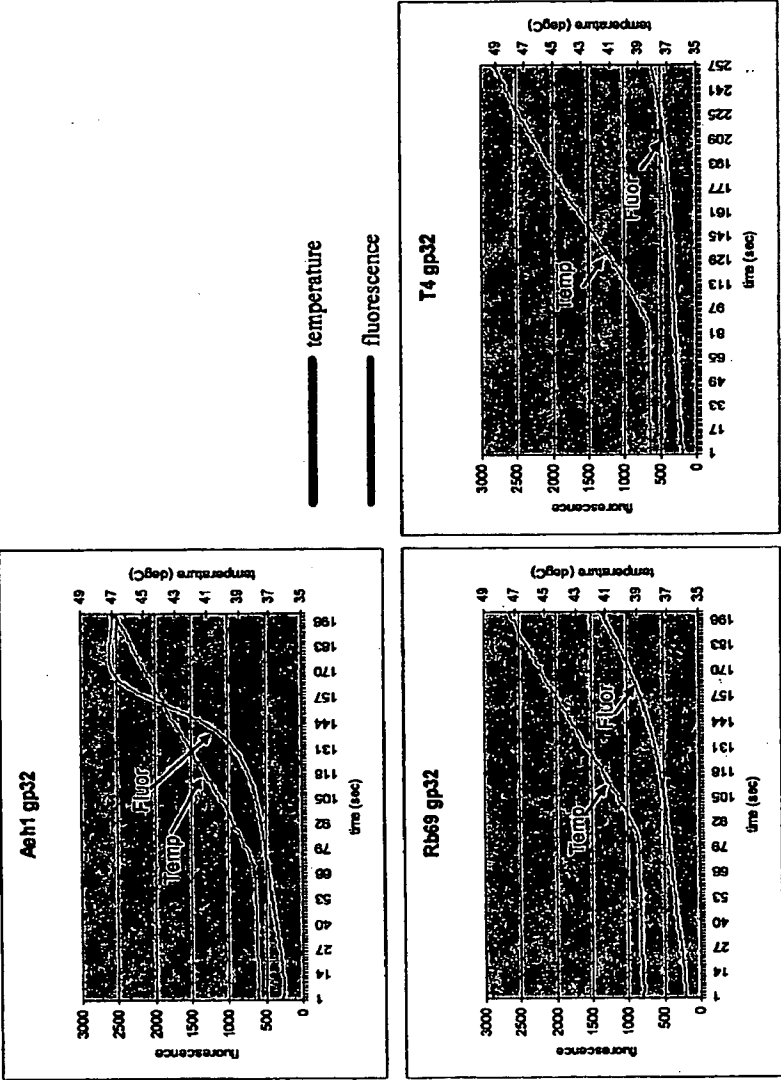


Figure 68

No DNA amplification in the absence of uvsY using T4 UvsX

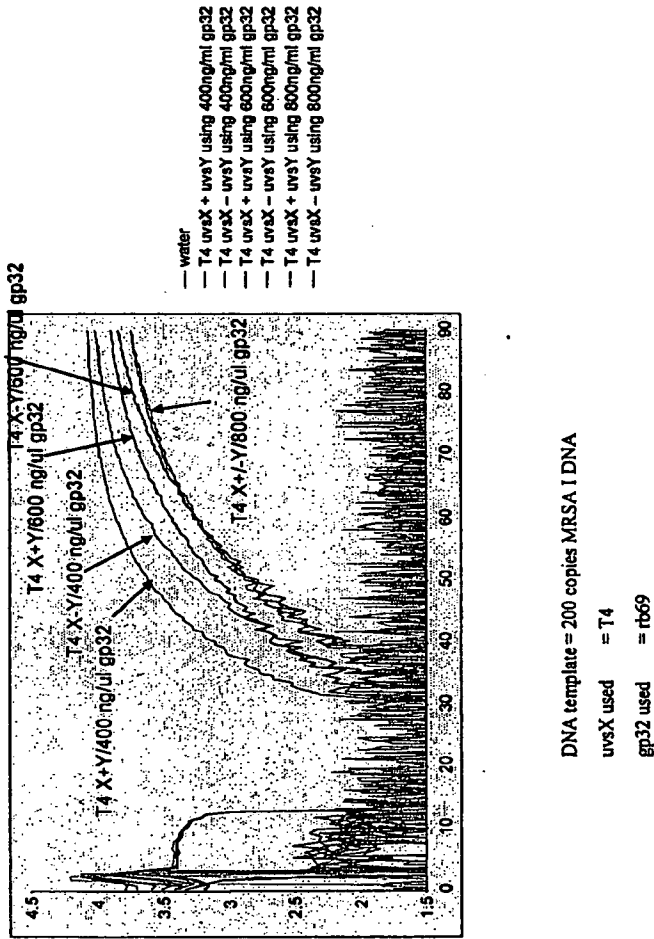


Figure 69

DNA amplification in the absence of uvsY using T4 UvsX

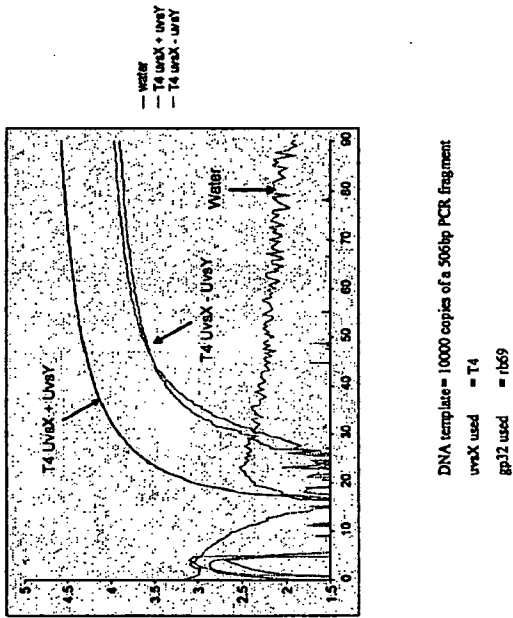


Figure 70

DNA amplification in the absence of uvsY using T4 UvsX

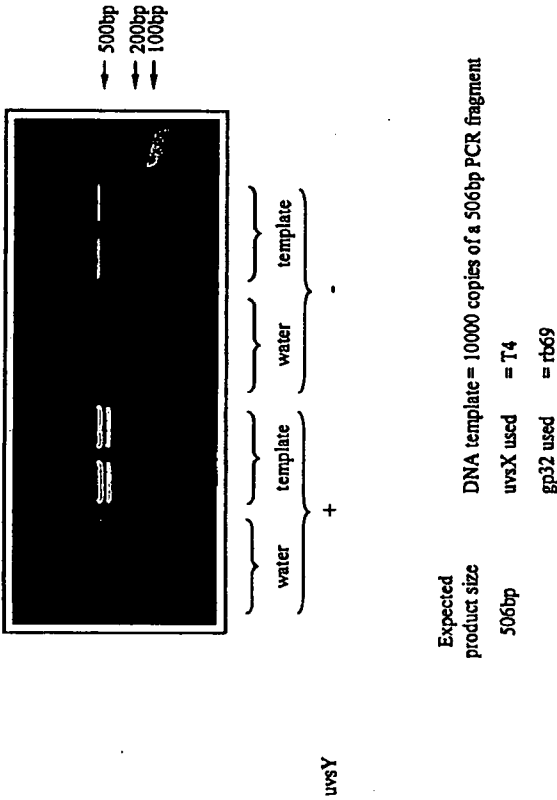


Figure 71