

## Standard Operation Procedure (S.O.P.) for Restriction Endonuclease Reaction

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This S.O.P. will direct you in the restriction endonuclease (restriction enzyme) reaction procedure so as to prepare cDNA and plasmids for subcloning or the linearization of an expression plasmid for the generation of cRNA through *in vitro* transcription kits for subsequent microinjection into *Xenopus laevis* oocytes. These enzymes recognize short and specific, often palindromic, double-stranded DNA sequence. They cleave within or adjacent to their recognition site. Restriction enzyme concentration is usually given in Units per mL. One unit is defined as the amount of enzyme that is required to digest 1 µg of lambda DNA (linear DNA obtained from the bacteriophage lambda) in 1 hour at 37 °C in a final volume of 50 µL.

Different factors could affect the restriction endonuclease reaction, including:

- Component storage temperature: restriction enzymes and their respective buffers must be kept at -20 °C.
- Conditions such as temperature, buffer composition, pH, ionic strength, and enzyme co-factors affect enzyme activity. Significant departure from ideal conditions can produce “*star activity*”, which results cleavages at non-canonical recognition sites or complete loss of specificity. Conditions that can lead to *star activity* are low ionic strength, high pH, and high glycerol concentrations (>5% v/v). Glycerol concentration is of particular interest, since commercial restriction enzymes are usually supplied in a buffer containing 50% of glycerol.
- Relative pure DNA is required for efficient restriction enzyme reaction. The DNA source may contain other types of DNA, nucleases, salts, and inhibitors of restriction enzymes.
- EDTA in low concentrations (1 mM) are usually used in the DNA storage buffer, such as TE (Tris-EDTA) buffer to prevent nuclease degradation of the DNA. EDTA can interference with the restriction enzyme reaction.
- Addition of acetylated Bovine Serum Albumin (BSA) or recombinant Albumin (rAlbumin) to a final concentration of 0.1 µg/µL has been shown to enhance the activity of restriction enzymes.
- A proper ratio of enzyme to DNA and Buffer is essential for the digestion. The restriction enzyme reaction is usually performed in a volume of 20–50 µL on 0.2–1.5 µg of DNA using a two- to ten-fold excess of enzyme Units. (e.g.: a ten-fold excess of enzyme for 1 µg of DNA is 10 Units).

### **Important:**

- **If the incubators and bath have not been recently used, check that the temperature is correctly set by using a calibrated thermometer**
- **See the Appendix to determine the buffer for restriction enzyme to be used.**
- **Enzyme should be the last component added to the reaction and should be taken from the freezer at the right moment of adding it to the reaction. *Keep the enzyme on ice all the time.***
- **If your DNA sample was storage in EDTA-containing buffers or you do not know the buffer of your sample, purify your DNA first and resuspend it in ultrapure water.**
- **Some enzymes could be heat inactivated at 65 °C. This procedure should be used to stop the restriction enzyme reaction when is available.**
- **If the volume that is needed for any of these reagents is less than 1 µL, a stock dilution should be prepared to add 1 µL of the reagent to the reaction tube.**
- **The rCutSmart and CutSmart buffers from New England BioLabs contain rAlbumin or BSA at 1 µg/µL, respectively.**

- **The temperatures for enzyme digestion and enzyme inactivation are usually 37 °C and 65 °C, respectively. Check the Enzyme Information Table in the Appendix for the appropriate temperatures.**

*Materials:*

- 70% Alcohol
- Micropipettes and Tips for 2, 10, and 20 (possible 100)  $\mu\text{L}$
- 0.5  $\mu\text{L}$  Microtubes
- Restriction Enzyme (store at  $-20\text{ }^{\circ}\text{C}$ )
- Reaction Buffer (10X; stored at  $-20\text{ }^{\circ}\text{C}$ )
- DNA To Be Digested (stored at 4 or  $-20\text{ }^{\circ}\text{C}$ )
- BSA at 10  $\mu\text{g}/\mu\text{L}$ , If Is Required (stored at  $-20\text{ }^{\circ}\text{C}$ )
- Nuclease-free Water (room temperature)
- Non-shaker Incubator at 37 °C
- Dry Bath at 65 °C (when required for heat inactivation)
- Microcentrifuge for 0.5  $\mu\text{L}$  Microtubes
- Ice Bucket
- Vortex

*Procedures:*

*Procedure 1:* Designed to set-up a restriction enzyme reaction for one enzyme in a volume of 50  $\mu\text{L}$ , 1  $\mu\text{g}$  of DNA (final concentration 0.02  $\mu\text{g}/\mu\text{L}$ ), and a five-fold excess of enzyme (5 Units). *To scale-up the reaction keep in mind that the final DNA concentration must be kept between (0.004–0.075  $\mu\text{g}/\mu\text{L}$ ) and a two- to ten-fold excess of enzyme Units must be used.*

- a) Equilibrate the non-shaker incubator at 37 °C and the dry bath (*if required*) at 65 °C for at least 30 minutes before placing the enzyme digestion reaction mixture. ***If the enzyme requires a different temperature for digestion and inactivation, adjust the temperature accordingly (see Appendix).***
- b) The working area should be sanitized using 70% ethanol prior to placing any materials on the bench.
- c) Make sure that the **Reaction Buffer** is properly thaw and mix before use.
- d) Calculate the volume of **Water**, **DNA**, **Reaction Buffer**, **BSA** (at 10  $\mu\text{g}/\mu\text{L}$ , *if required*), and **Restriction Enzyme** that needs to be used.
  - ***In the case that a stock dilution is required, the DNA could be diluted in water, but the stock dilution for BSA or the enzyme must be prepared to a final 1X buffer concentration.*** Take the buffer concentration in consideration when assembling the reaction (e.g.: if you are assembling a 20  $\mu\text{L}$  reaction and needs to dilute the BSA to 2  $\mu\text{g}/\mu\text{L}$  (buffer 1X) to add 1  $\mu\text{L}$  to the reaction, then the buffer calculation for the rest of the reaction should be based on 19  $\mu\text{L}$ ).
- e) Add the required volume of **Water**.
- f) Add 1 $\mu\text{g}$  of **DNA** and mix by pipetting up and down.
- g) Add the **Reaction Buffer** to a final concentration of 1X and mix by pipetting up and down.
- h) If necessary, add **BSA** to a final concentration of 0.1  $\mu\text{g}/\mu\text{L}$ .

- i) Spin-down the reaction in a microcentrifuge and vortex to ensure proper mixing.
- j) Add 5 Units of the **Restriction Enzyme** and mix by pipetting up and down.
- k) Incubate for 6 hours in the non-shaker incubator (usually 37 °C).
- l) *Optional*: Incubate the digestion reaction in the dry bath (usually 65 °C) for the appropriate time (see Appendix) if the enzyme could be inactivated.
- m) Decontaminate the work area with 70% ethanol at the end.
- n) Be sure all equipment are shut down: incubator and dry bath. Return any material and equipment to its proper place. The pipettes must be returned to their maximal volume.
- o) Analyze 50 ng of DNA through agarose gel electrophoresis.
- p) The rest of the reaction can be purified if necessary. Possible kits to use are Promega Wizard Clean-Up System (Cat. No. A7280), Promega Wizard SV Gel and PCR Clean-Up System (Cat. No. A9281), Qiagen QIAquick Spin Column (Cat. No. 28115), and Qiagen QIAquick Gel Extraction Kit (Cat. No. 28704).

***Procedure II***: Designed to set-up two sequential restriction enzyme reactions in a volume of 20  $\mu\text{L}$ , 1  $\mu\text{g}$  of DNA (final concentration 0.05  $\mu\text{g}/\mu\text{L}$ ), a five-fold excess of the enzyme (5 Units) used in the first reaction, and a two-fold excess of the enzyme (2 Units) used in the second reaction. *To scale-up the reaction keep in mind that the final DNA concentration must be kept between (0.004–0.075  $\mu\text{g}/\mu\text{L}$ ) and a two- to ten-fold excess of enzyme Units must be used. Use enzymes from the same company to avoid additional purification steps.*

- a) Select the best buffer for the restriction enzyme reaction. Use a buffer with 100% of activity in both enzymes; preferably, the universal buffers offered by companies, such as MultiCore Buffer for Promega and CutSmart for New England Biolabs. *If a buffer with 100% of activity in both enzymes cannot be found, select the buffer with the highest activity combine; preferably, with 100% of activity in one enzyme.*
- b) Select enzymatic reaction will be performed first. Preferably, an enzyme that can be 100% digested in the selected buffer and can be inactivated.
- c) Equilibrate the non-shaker incubator at 37 °C and the dry bath (*if required*) at 65 °C for at least 30 minutes before placing the enzyme digestion reaction mixture. ***If the enzyme requires a different temperature for digestion and inactivation, adjust the temperature accordingly (see Appendix).***
- d) The working area should be sanitized using 70% ethanol prior to placing any materials on the bench.
- e) Make sure that the **Reaction Buffer** is properly thaw and mix before use.
- f) Calculate the volume of **Water**, **DNA**, **Reaction Buffer**, **BSA** (at 10  $\mu\text{g}/\mu\text{L}$ ), and **Restriction Enzyme** that needs to be used.
  - o ***If the volume that is needed for any of these reagents is less than 1  $\mu\text{L}$ , a stock dilution should be prepared to add 1  $\mu\text{L}$  of the reagent to the reaction tube. The stock dilution for DNA could be diluted in water, but the stock dilution for BSA or the enzyme must be prepared to a final 1X buffer concentration.*** Take the buffer concentration in consideration when assembling the reaction (e.g.: if you are assembling a 20  $\mu\text{L}$  reaction and needs to dilute the BSA to 2  $\mu\text{g}/\mu\text{L}$  (buffer 1X) to add 1  $\mu\text{L}$  to the reaction, then the buffer calculation

for the rest of the reaction should be based on 19  $\mu\text{L}$ .

- g) Add the required volume of **Water**.
- h) Add 1 $\mu\text{g}$  of **DNA** and mix by pipetting up and down.
- i) Add the **Reaction Buffer** to a final concentration of 1X and mix by pipetting up and down.
- j) If necessary, add **BSA** to a final concentration of 0.1  $\mu\text{g}/\mu\text{L}$ .
- k) Spin-down the reaction in a microcentrifuge and vortex to ensure proper mixing.
- l) Add 5 Units of the first **Restriction Enzyme** and mix by pipetting up and down.
- m) Incubate for 6 hours in the non-shaker incubator (usually 37  $^{\circ}\text{C}$ ).
- n) Inactivate the digestion reaction in the dry bath (usually 65  $^{\circ}\text{C}$ ) for the appropriate time and remove 1  $\mu\text{L}$  of the reaction for further analysis.
  - o If inactivation is not possible, purify the DNA using one of the DNA clean-up systems described in *Procedure I, Step m*). Assemble the second reaction as described in *Steps e–m* and proceed to *Step q*.
- o) Add 2 Units of the second **Restriction Enzyme** to the digestion reaction mixture.
- p) Spin-down the reaction in a microcentrifuge and vortex to ensure proper mixing.
- q) Incubate overnight in the non-shaker incubator (usually 37  $^{\circ}\text{C}$ ).
- r) Decontaminate the work area with 70% ethanol at the end.
- s) Be sure all equipment are shut down: dry bath. Return any material and equipment to its proper place. The pipettes must be returned to their maximal volume
- t) Inactivate the digestion reaction the next day if possible. Separate the DNA bands through agarose gel electrophoresis and purify the band of interest using a gel extraction system, such as Promega Wizard SV Gel and PCR Clean-Up System (Cat. No. A9281) or Qiagen QIAquick Gel Extraction Kit (Cat. No. 28704).

*Appendix:*

**List of Restriction Enzymes**

<b>Enzyme</b>	<b>Company</b>	<b>Reaction Buffer (100% Efficiency)</b>	<b>Universal Buffer (% Efficiency)</b>	<b>Heat Inactivation (Temp; Time)</b>	<b>Star Activity</b>
BamHI	Promega	Buffer E	MULTI-CORE (75–100%)	Yes (65 °C; 20 min)	No
XhoI	New England BioLabs	rCutSmart	rCutSmart (100%)	Yes (65 °C; 20 min)	No
MluI-HF	New England BioLabs	rCutSmart	rCutSmart (100%)	No	No
HindIII	Promega	Buffer E	MULTI-CORE (50–75%)	Yes (65 °C; 20 min)	No
EcoRI	Promega	Buffer H	MULTI-CORE (100%)	Yes (65 °C; 20 min)	No
NotI-HF	New England BioLabs	rCutSmart (100%)	rCutSmart (100%)	Yes (65 °C; 20 min)	No