5BBG0206 Molecular Biology Research Skills 2021-2022

Experimental design proforma.

Construction and use of expression vector pFXN-AcGFP1-N1 encoding Frataxin with C-terminal GFP fusion

Part 1. Design gene specific part of the forward and reverse primers to amplify the coding region of human frataxin, isoform 1 preproprotein for use to create C-terminal fusion with GFP (10 marks)

Figure 1. Part of the DNA sequence of human frataxin, isoform 1 preproprotein cDNA insert in pOTB7 (IMAGE:4842134) translated using EMBL EBI EMBOSS Sixpack programme to show the three forward open reading frames from nucleotide 1 to 720. The frataxin preproprotein amino acid sequence is highlighted yellow, the DNA sequence of the gene specific part of the forward primer is highlighted in red, the DNA sequence of the gene specific part of the reverse primer is highlighted in pink.

```
   1 gcggagcgggcggcagacccggagcagcatgtggactctcgggcgccgcgcagtagccgg 60
     ----:----|----:----|----:----|----:----|----:----|----:----|
   1 cgcctcgcccgccgtctgggcctcgtcgtacacctgagagcccgcggcgcgtcatcggcc 60
     A  E  R  A  A  D  P  E  Q  H  V  D  S  R  A  P  R  S  S  R     F1
      R  S  G  R  Q  T  R  S  S  M  W  T  L  G  R  R  A  V  A  G    F2
       G  A  G  G  R  P  G  A  A  C  G  L  S  G  A  A  Q  *  P  A   F3

  61 cctcctggcgtcacccagcccggcccaggcccagaccctcacccgggtcccgcggccggc 120
     ----:----|----:----|----:----|----:----|----:----|----:----|
  61 ggaggaccgcagtgggtcgggccgggtccgggtctgggagtgggcccagggcgccggccg 120
     P  P  G  V  T  Q  P  G  P  G  P  D  P  H  P  G  P  A  A  G     F1
      L  L  A  S  P  S  P  A  Q  A  Q  T  L  T  R  V  P  R  P  A    F2
       S  W  R  H  P  A  R  P  R  P  R  P  S  P  G  S  R  G  R  Q   F3

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     ----:----|----:----|----:----|----:----|----:----|----:----|
 121 tctcaaccggggtgagacgccggcggcaccggacgcgtggctgtagctacgctggacgtg 180
     R  V  G  P  T  L  R  P  P  W  P  A  H  R  H  R  C  D  L  H     F1
      E  L  A  P  L  C  G  R  R  G  L  R  T  D  I  D  A  T  C  T    F2
       S  W  P  H  S  A  A  A  V  A  C  A  P  T  S  M  R  P  A  R   F3

 181 gccccgccgcgcaagttcgaaccaacgtggcctcaaccagatttggaatgtcaaaaagca 240
     ----:----|----:----|----:----|----:----|----:----|----:----|
 181 cggggcggcgcgttcaagcttggttgcaccggagttggtctaaaccttacagttttcgt 240
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      P  R  R  A  S  S  N  Q  R  G  L  N  Q  I  W  N  V  K  K  Q    F2
       P  A  A  Q  V  R  T  N  V  A  S  T  R  F  G  M  S  K  S  R   F3

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     ----:----|----:----|----:----|----:----|----:----|----:----|
 241 ctcacagataaactacttaaactcctttagaccttgaaacccggtgggtccgagagatct 300
     E  C  L  F  D  E  F  E  E  I  W  N  F  G  P  P  R  L  S  R     F1
      S  V  Y  L  M  N  L  R  K  S  G  T  L  G  H  P  G  S  L  D    F2
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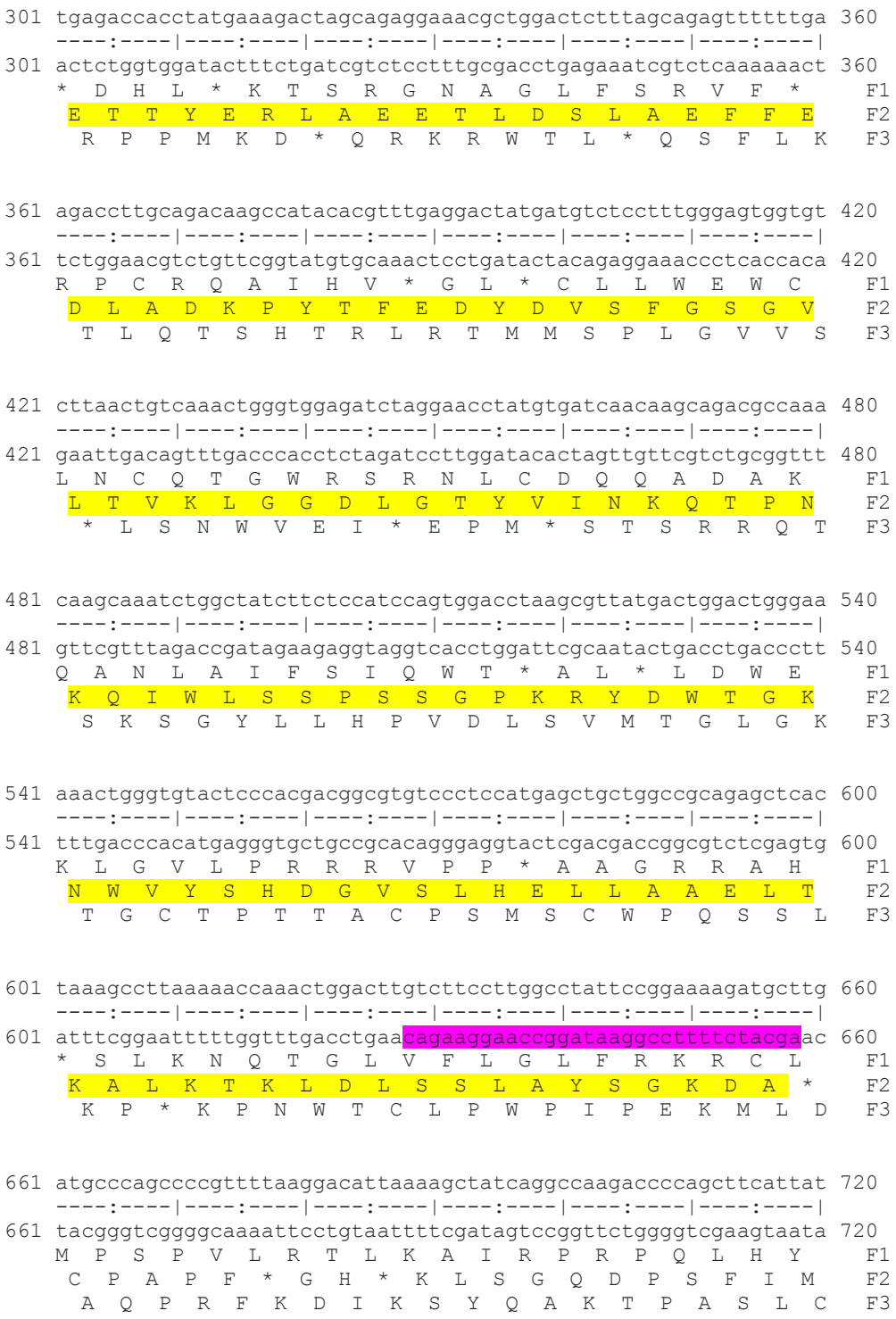
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* D H L * K T S R G N A G L F S R V F *
E T T Y E R L A E E T L D S L A E F F E
R P P M K D * Q R K R W T L * Q S F L K
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D L A D K P Y T F E D Y D V S F G S G V
T L Q T S H T R L R T M M S P L G V V S
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L N C Q T G W R S R N L C D Q Q A D A K
L T V K L G G D L G T Y V I N K Q T P N
* L S N W V E I * E P M * S T S R R Q T
481 caagcaaatctggctatcttctccatccagtggacctaagcgttatgactggactgggaa 540
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Q A N L A I F S I Q W T * A L * L D W E
K Q I W L S S P S S G P K R Y D W T G K
S K S G Y L L H P V D L S V M T G L G K
541 aaactgggtgtactcccacgacggcgtgtccctccatgagctgctggccgcagagctcac 600
541 tttgacccacatgagggtgctgccgcacagggaggtactcgacgaccggcgtctcgagtg 600
K L G V L P R R R V P P * A A G R R A H
N W V Y S H D G V S L H E L L A A E L T
T G C T P T T A C P S M S C W P Q S S L
601 taaagccttaaaaaccaaactggacttgtcttccttggcctattccggaaaagatgcttg 660
601 atttcggaatttttggtttgacctgaacagaaggaaccggataaggccttttctacgaac 660
* S L K N Q T G L V F L G L F R K R C L
K A L K T K L D L S S L A Y S G K D A *
K P * K P N W T C L P W P I P E K M L D
661 atgcccagccccgttttaaggacattaaaagctatcaggccaagaccccagcttcattat 720
661 tacgggtcggggcaaaattcctgtaattttcgatagtccggttctggggtcgaagtaata 720
M P S P V L R T L K A I R P R P Q L H Y
C P A P F * G H * K L S G Q D P S F I M
A Q P R F K D I K S Y Q A K T P A S L C

Table 1. Frataxin gene specific DNA sequences of forward and reverse primers and Tm values

	DNA sequence	Tm
Forward Primer	atgtggactctcgggcgccgcgcag	67.5^{o}C
Reverse Primer	agcatcttttccggaataggccaaggaagac	63^{o}C

Table 2. Predicted size of PCR product using gene specific forward and reverse primers from Table 1

Predicted size of DNA fragment	630 bp

Table 3. PCR cycling parameters

Segment	Number of cycles	Temperature	Duration
1	1	95°C	2 minutes
2	30	95°C	20 seconds
		58°C	20 seconds
		72°C	15 seconds
3	1	72°C	3 minutes

Part 2. Sub-cloning the human frataxin preproprotein gene in-frame with N-terminus of the green fluorescent protein in pAcGFP1-N1 to create a plasmid called pFXN-AcGFP1-N1 (30 marks)

Table 4. Choosing restriction enzymes to directionally sub-clone human frataxin preproprotein gene into pAcGFP1-N1

Promega Restriction Enzyme	Recognition sequence	Recognition sequence in pAcGFP1-N1 MCS (yes/no)	Recognition sequence in frataxin preproprotein coding sequence (yes/no)	Promega buffers (100% activity)
*Age*I	A↓CCGGT	Yes	No	K, Multicore
*Apa*I	GGGCC↓C	Yes	No	A
*Bam*HI	G↓GATCC	Yes	No	E
*Bgl*II	A↓GATCT	Yes	Yes	D
*Eco*RI	G↓AATTC	Yes	No	H
*Hind*III	A↓AGCTT	Yes	No	B, E
*Kpn*I	GGTAC↓C	Yes	No	J
*Nhe*I	G↓CTAGC	Yes	No	B, Multicore
*Pst*I	CTGCA↓G	Yes	No	H
*Sac*I	GAGCT↓C	Yes	Yes	E, Multicore, J
*Sac*II	CCGC↓GG	Yes	Yes	A, H, C
*Sma*I	CCC↓GGG	Yes	Yes	Multicore, J
*Xho*I	C↓TCGAG	Yes	No	H, D

Table 5. Possible pairs of restriction enzymes that can added to the forward and reverse PCR primers to sub-clone clone human frataxin preproprotein gene into pAcGFP1-N1

	Restriction enzyme forward primer	Restriction enzyme reverse primer	Optimum buffer for 100% activity
1	*Xho*I	*Pst*I	H
2	*Nhe*I	*Age*I	Multicore
3	*Hind*III	*Bam*HI	E
4	*Nhe*I	*Hind*III	B
5	*Xho*I	*Eco*RI	H

Table 6. Full DNA sequences of final forward and reverse primers (frataxin specific sequence highlighted yellow, *Xho*I recognition sequence highlighted red, *Pst*I recognition sequence highlighted pink, other additional nucleotides highlighted blue).

	DNA sequence
Forward Primer	gggctcgagatgtggactctcgggcgccgcgcag
Reverse Primer	gggctgcagagcatcttttccggaataggccaaggaagac

Figure 2. DNA sequence of pFXN-AcGFP1-N1 (frataxin specific sequence highlighted yellow, AcGFP1-N1 sequence highlighted green, *Xho*I recognition sequence highlighted red, *Pst*I recognition sequence highlighted pink).

TAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACAT
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GTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAA
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Figure 3. Plasmid map of pFXN-AcGFP1-N1 taken from SnapGene.

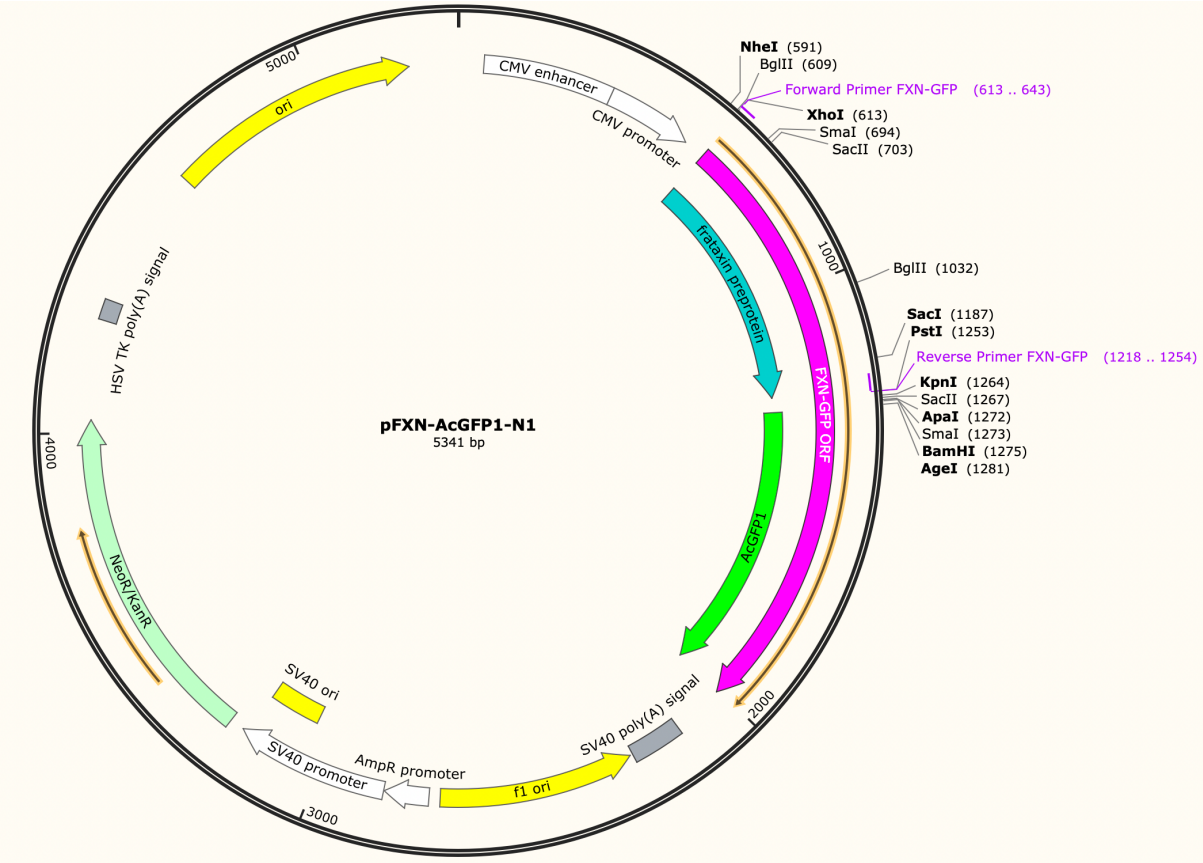


Figure 4. Nucleotide and amino acid sequence showing the start of the FXN-GFP open reading frame and the junction between frataxin preproprotein and pFXN-AcGFP1-N1 taken from SnapGene.

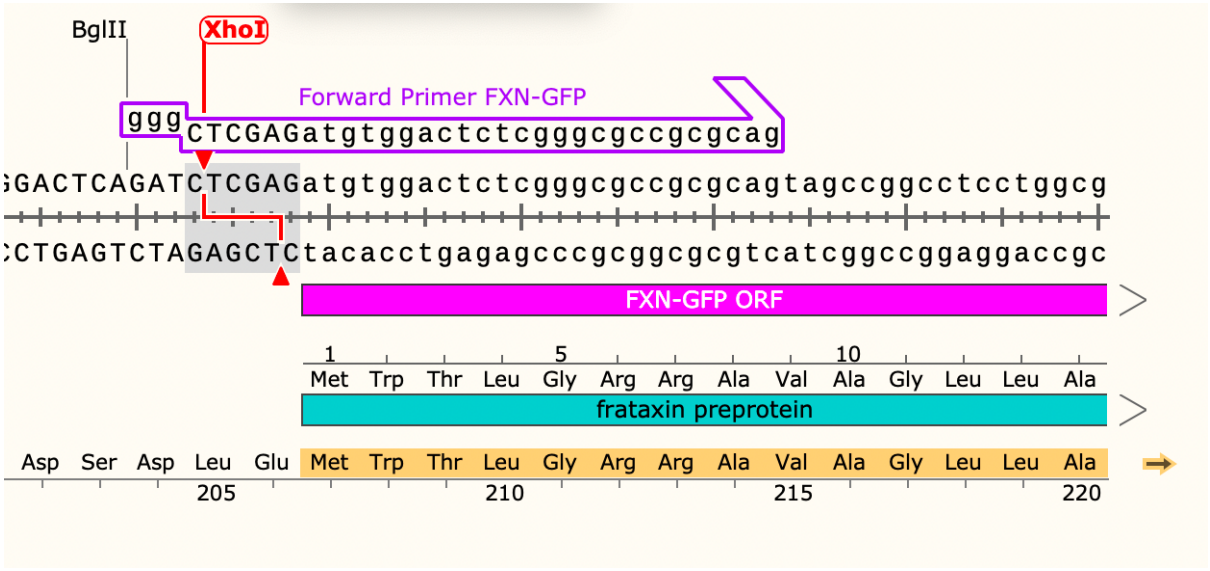


Figure 5. Nucleotide and amino acid sequence showing the junction between C terminal amino acids of frataxin preproprotein and the N-terminal amino acids of green fluorescent protein in pFXN-AcGFP1-N1 taken from SnapGene.

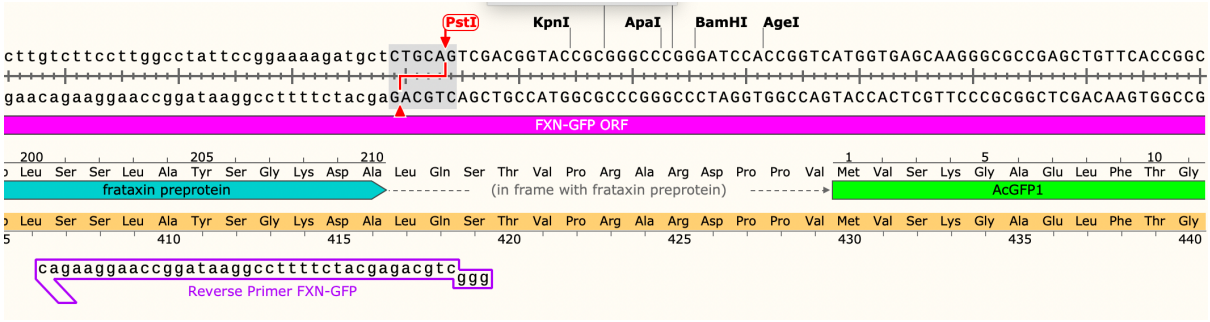
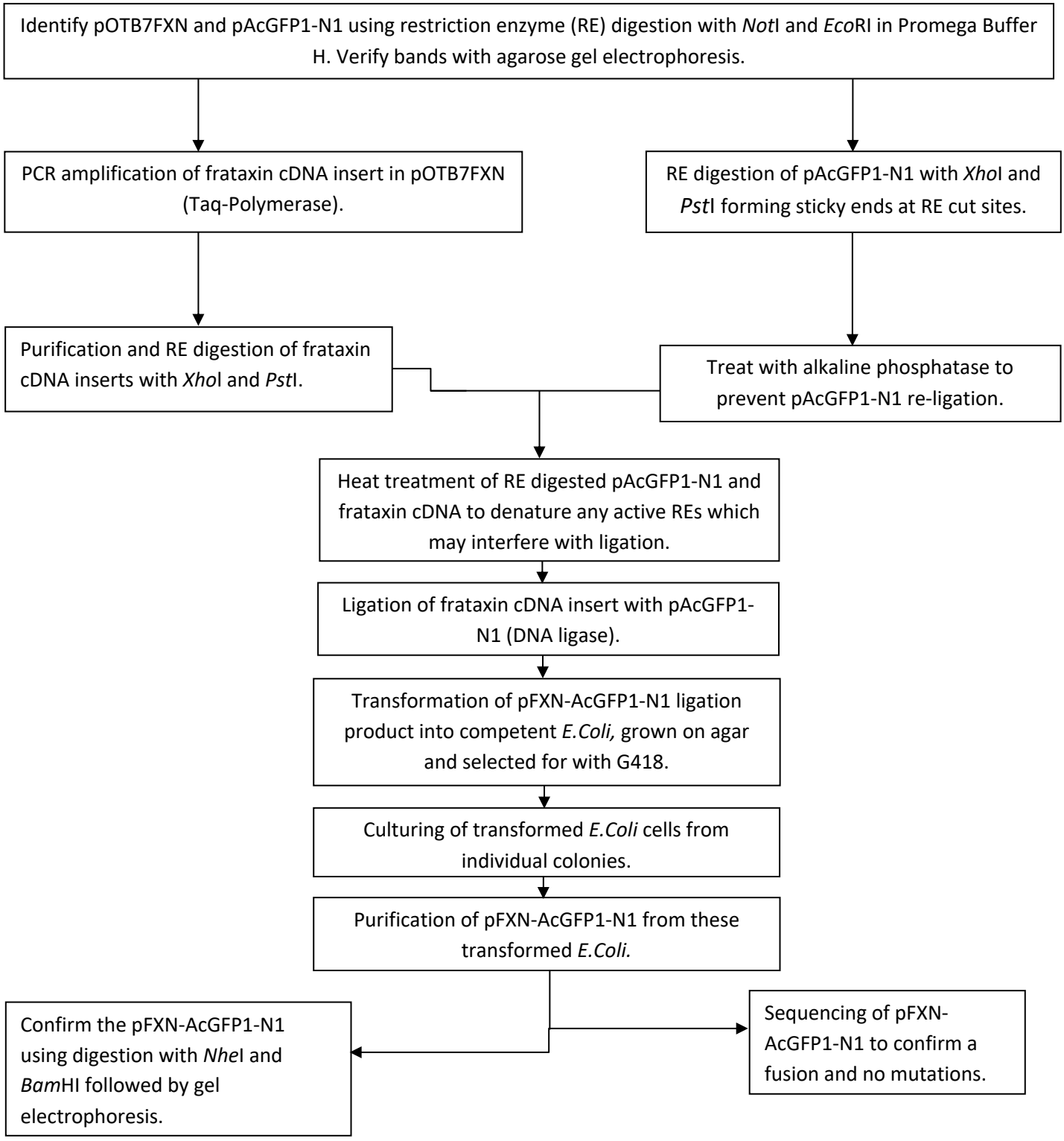


Figure 6. The full amino acid sequence of the FXN-GFP open reading frame from SnapGene pFXN-AcGFP1-N1 with frataxin preproprotein amino acids highlighted yellow, GFP amino acids highlighted green, and any other additional amino acids highlighted blue).

MWTLGRRAVAGLLASPSPAQAQTLTRVPRPAELAPLCGRRGLRTDIDATCTPRRASSNQRGLNQIW NVKKQSVYLMNLRKSGTLGHPGSLDETTYERLAEETLDSLAEFFEDLADKPYTFEDYDVSFGSGVLTVKL GGDLGTYVINKQTPNKQIWLSSPSSGPKRYDWTGKNWVYSHDGVSLHELLAAELTKALKTKLDLSSLA YSGKDALQSTVPRARDPPVMVSKGAELFTGIVPILIELNGDVNGHKFSVSGEGEGDATYGKLTLKFICTT GKLPVPWPTLVTTLSYGVQCFSRYPDHMKQHDFFKSAMPEGYIQERTIFFEDDGNYKSRAEVKFEGDT LVNRIELTGTDFKEDGNILGNKMEYNYNAHNVYIMTDKAKNGIKVNFKIRHNIEDGSVQLADHYQQN TPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMIYFGFVTAAAITHGMDELYK*

Part 3. Experimental steps required to construct pFXN-AcGFP1-N1 using standard molecular biology techniques (20 marks)

Figure 6. Flow diagram showing experimental steps required to construct pFXN-AcGFP1-N1



Words: 149

Part 4. *In vivo* experimental design. Transfection of pFXN-AcGFP1-N1 into HeLa tissue culture cells. (40 marks)

In this experiment we will be transfecting a pFXN-AcGFP-N1 plasmid into human HeLa tissue cells (grown in tissue culture medium and maintained in EMEM buffer) to study the intracellular location of frataxin [*ATCC*, 2021]. Transfection can be non-viral: chemical techniques are lipid-mediated or calcium phosphate co-precipitation, but physical techniques involve electroporation neon transfection systems which we will use in-vivo to introduce 1-5ug of pFXN-AcGFP-N1 DNA into our 10^6 HeLa cell sample [*Thermofisher*, 2021].

We first trypsinise and harvest our HeLa cells by centrifugation, ready to be measured out in 0.5ml aliquots, suspended in conductive electroporation buffer and mixed with pFXN-AcGFP-N1 in cuvettes on ice. After 5 minutes incubation, electrical pulses (pulse width is determined by capacitance and must maintain 40-80% survival of cells) produce voltage which develop temporary pores in the HeLa cell's phospholipid bilayer: passages to insert our DNA using an electronic pipette tip. The transfected cells are selected for in a medium with G418 as the pFXN-AcGFP-N1 CMV promotor regulates the neomycin resistance gene; successful colonies are then re-cultured at 37^{o}C incubation for 50-60 hours for transient expression [*Thermofisher*, 2016][Damdindorj *et al.,* 2014][Potter and Heller, 2003].

Since pAcGFP-N1 is an N-terminal specification, the GFP coding sequence is outside the MCF and the Frataxin-GFP fusion protein produced by pFXN-AcGFP-N1 has GFP at its C-terminal. Fluorescence microscopy under UV at 475nm can be used to observe GFP emission at 505nm and localise Frataxin-GFP produced constitutively (via the CMV promotor) by transfected HeLa cells [Damdindorj *et al.,* 2014][*Takarabio*, 2022]. The intracellular location of FXN-GFP will be regulated by the frataxin as it is the N-terminal component where signal peptides are located [Palmer and Freeman, 2004]. Based on frataxin studies, the location of fluorescence is expected to be in the mitochondrial protein and iron-sulphur clusters due to its proposed function as an iron chaperone [Adinolfi *et al.,* 2009].

We could further study the relationship between reduced frataxin expression and Friedrich's Ataxia, the effect of various drugs on frataxin concentration in-vivo or changes in extra-mitochondrial frataxin localisation during differentiation: e.g., in enterocyte-like Caco-2 cells [Acquaviva *et al.,* 2005]. For long term studies we can create a stable HeLa cell line by passaging the cells over weeks or months of selection for a colony with the plasmid DNA construct integrated into a chromosome. Transfection can also be used for RNAi studies controlling gene expression with siRNA to treat genetic diseases [Kim and Eberwine, 2010].

Words: 400

References

Acquaviva, F., De Biase, I., Nezi, L., Ruggiero, G., Tatangelo, F., Pisano, C., Monticelli, A., Garbi, C., Acquaviva, A. M., & Cocozza, S. (2005). Extra-mitochondrial localisation of frataxin and its association with IscU1 during enterocyte-like differentiation of the human colon adenocarcinoma cell line Caco-2. *Journal of cell science*, *118*(Pt 17), 3917–3924. https://doi.org/10.1242/jcs.02516

Adinolfi, S., Iannuzzi, C., Prischi, F., Pastore, C., Iametti, S., Martin, S. R., Bonomi, F., & Pastore, A. (2009). Bacterial frataxin CyaY is the gatekeeper of iron-sulfur cluster formation catalyzed by IscS. Nature structural & molecular biology, 16(4), 390–396. https://doi.org/10.1038/nsmb.1579

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