# HD Resolution Lab Notebook

**Project:** iGEM Notebook Final
**Authors:** HD Resolution iGEM
**Dates:** 2017-09-18 to 2017-10-30

MONDAY, 9/18/17

## Creating Reagents for Separating Plasmid DNA

1. Prepare 100 mL of TE Buffer 10X; 10 mM Tris-Cl, 10 mM EDTA, and fill with dH2O upto 100 mL.
2. Prepare 1 L of Alkaline Lysis Solution I: 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, and fill with dH2O upto 1 L.
3. Prepare 1.2 L of Alkaline Lysis Solution III: 600 mL of 2M Potassium Acetate, 115 mL of glacial acetic acid, 285 mL of H2O.
4. Prepare 0.5 L of Alkaline Lysis Solution II: Making Alkaline Lysis Buffer

TUESDAY, 9/19/17

## Cell Competency Testing Day 2

Clean working area with 10% ethanol

1. Throw competent cells on ice
2. Label one 1.5 mL microcentrifuge tubes for each transformation and pre-chill on ice.
    a. Orange = 50 pg/µl
    b. Yellow = 10 pg/µl
    c. Red = 100 pg/µl
3. Spin down DNA tubes from competent cell test kit. Quick spin of 20-30 seconds at 8000-10000rpm.
4. Pipet 10µl of DNA into centrifuge tubes. 50µl of competent cells already aliquoted. Flick gently.
5. Incubate on ice for 30 min.
6. Heatshock cells into water bath of 42 degrees Celsius for 45 sec.
7. Immediately transfer tubes back into ice and incubate on ice for 5 min.
8. Add 950 µl of SOC Media per tube and incubate at 37 degrees Celsius for 1 hour, shaking at 200-300rpm.
9. Pipet 100µl from each tube onto appropriate plate, spread mixture evenly.
10. Incubate at 37 degrees Celsius overnight/16 hrs. Place plates with agar side on top and lid on bottom.

WEDNESDAY, 9/20/17

## Cell Competency Testing Day 3

1. Innoculated 600 mL LB w/ 1 mL O/N culture.
2. Shake at 37 degrees Celsius, DDC 600 = 0.4, 3 hr.
3. Cooled all materials in ice, including solutions (CaCl3), cells, tubes, & tips.
4. Aliquoted 600 mL of cells into 12 50 mL tubes, 30 mL each.
5. Spin 1.5 k rcf, 5 min.
6. Decant sup, resus, pellets in 1 mL CaCl2 (cold) by gentle rocking, left on ice for 30 min.
7. Spin as above.
8. Discard supernatant.
9. Added fresh cold 100 mM CaCl3.
10. Resuspend as above.
11. Added 10.2 mL 50% glycerol to 50 mL falcon tube (chilled).
12. Consolidated all resuspended cells into the tube containing 50% glycerol.
13. Did not fully resuspend cells, still looked chunky.
14. Froze in 1 mL aliquots of liquid nitrogen, left in unmarked box below iGEM box in -80 degrees Celsius freezer.

FRIDAY, 9/22/17

## Continuation

1. Added 175µl of ice cold Alkaline Lysis Solution III through the viscous bacterial lysate. Inverted tube several times and stored tube on ice for 5 min.
2. Centrifuged lysate at maximum speed for 5 min at 4 degrees Celsius, and transferrred the supernatant to a new tube.
3. Precipitated the nucleic acids from the supernatant. Added 1 mL of ethanol at room temperature. Mixed solution by vortexing and allowed mixture to stand for two minutes at room temp.
4. Collected precipitated nucleic acds by centrifugation at max speed for 5 min at 4 degrees Celsius. Dropped precipitated nucleic aicds. Redid centrifugation at max spped for 5 minutes at 4 degrees Celsius.
5. Removed supernatant by gentle aspiration, as shown in step 4. Stood tube in an inverted position on a paper towel to allow all the fluid to drain away. Used a Kimwipe to remove the drops of fluid adhering to the walls of the tube.
6. Added 1 mL of 70% ethanol to the pellet and inverted the closed tube several times.
7. Recovered DNA by centrifugation at max speed for 2 min at 4 degrees Celsius in a microcentrifuge.

SATURDAY, 9/23/17

## Mini-Prep

1. Pelleted 40261, 40262, DH5 x cells at 800 rcf, 10 minutes at 4 C.
2. Meanwhile, prepared 1 % agarose gel.
    1 g of agarose
    10 mL of 10X TAE
    10 mL of dH2O
    5 uL of EtBr
3. Centrifuge tubes at 2,000 rcf for 10 minutes. Decant supernatant.
4. Add 250 uL of Resuspension Solution and vortex.
5. Add 250 uL of Lysis solution and invert 4-6 times.
6. Add 350 uL of Neutralization solution and invert 4-6 times.
7. Centrifuge at 12,000 rcf for 5 minutes.
8. Transfer supernatant to a GeneJET Spin Column.
9. Centrifuge at 12,000 rcf for 1 minute.
10. Add 500 uL of Wash Solution and centrifuge for 30 seconds at 12,000 rcf. Discard flow through.
11. Repeat step 10.
12. Transfer column to a new Eppendorf tube and add 15 uL of Elution Buffer to column and incubate at room temperature for 2 minutes.
13. Centrifuge at 12,000 rcf for 2 minutes.
14. Collect flow through, containing the bacterial plasmid.

MONDAY, 9/25/17

## Transformation

1. Thaw DHS-Alpa Cells on ice.
2. Add 2 mL of DNA to cells (from previously isolated adgene plasmids).
3. Stand for 34 min on ice.
4. Cells are incubated for 60 sec at 42 degrees Celsius.
5. Put cells on ice for 5 minutes.
6. Add 900µl SOC media.

TUESDAY, 9/26/17

## Interlab and Second Cell Competency Test

1. Clean working area.
2. Thaw competent cells on ice.
3. Label 8 microcentrifuge tubes : 10ug, 50ug, 100ug, 1, 4, 5, 6, Neg.
4. Spin down DNA from competent cell test kit and interlab DNA.
5. Pipet 1µl of DN from competent cell test kit into tubes labeled 1, 4, 5, 6, neg.
6. Pipet 85µl of competent cells into all tubes, flick gently to mix.
7. Incubate on ice for 30 minutes.

8. Heat shock cells by placing tubes into a hot water bath at 42 degrees Celsius for 45 seconds.
9. Transfer back and  incubate on ice for 5 min.
10. Add 45µl of SOC Media into eah cand incubate at 37 degrees for 1 hour shaking at 200-300 rpm.
11. Warm up agar plates, label.
12. Pipet 100µl from each tube onto appropriate plate, spread mixture evenly across plates via sterile glass beads.
13. Close with parafilm.
14. Incubate at 37 degrees Celsius overnight with plates positioned with agar side at top and lid at bottom.

## Preparation of LB-Agar Plates

Preparation of LB-Agar Plates
- Adapted from CSH Protocols

LB Agar
- 3.75 gramf of Agar
- 250 mL of 1x LB Medium

1. Mix
2. Autoclave on Liquid Cycle for 30 minutes.
    a. Do not overfill bottles.
        I. 250 mL of LB was autoclaved in a 500 mL bottle to prevent it from boiling over.
3. Add 2.5 mL of Kanamycin Stock (25 mg/mL) to a final concentration of 25ug/mL, once LB-Agar has coded to about 50 degrees Celsius.
4. Heat on a hot plate to re-melt sugar.

## Running Gel

1. Pour gels as per protocol Casting Gel for Electrophoresis
2. Add 6X loading dye to each sample.
    a. 10µL per sample
3. Load gel with 2-log ladder and samples.
4. Run electrophoresis at 100V for 30 minutes.

MONDAY, 10/2/17

## Mini-Prep (AddGene Plasmids)

1. Create 1 mL of Alkaline Lysis Solution II: Making Alkaline Lysis Buffer
2. Pour 5 mL of culture into microcentrifuge tube, spin at max speed for 30 seconds at 4 C (divided into 3 tubes each).
3. Remove media with aspiration, leaving pellet as dry as possible.
4. Resuspend pellet in 100 uL of ice-cold Alkaline Lysis Solution I with vigorous vortexing (combined tubes into 1 tube each).
5. Add 450 uL of fresh Alkaline Lysis Solution II to each bacterial suspension. Invert rapidly 5 times. Do not vortex. Place tubes on ice.
6. Add 450 uL of ice-cold Alkaline Lysis Solution III, invert several times. Store on ice for 5 minutes.
7. Centrifuge at max speed for 5 minutes at 4 C, transfer supernatant to a fresh tube.
8. Add 2 volumes (950 uL) of ethanol at room temp. Vortex and allow mixture to stand for 2 minutes at room temp.
9. Centrifuge at max speed for 5 minutes at 4 C.
10. Remove supernatant by aspiration. Use kimwipe to remove any drops of fluid adhering to walls of tube.
11. Add 1 mL of 70% ethanol to pellet and invert several times. Centrifuge at max speed for 2 minutes at 4 C.
12. Remove supernatant with aspiraton.

TUESDAY, 10/3/17

## Cut out Exon 1 from AddGene and Verify Cuts

1. Use Nanodrop blanked with 10X TE Buffer.
2. Measure A 40261, A 40262, OCT 40261-A, OCT 40262-A ng/mL

    A 40261 = 92.9 ng/mL

    A 40262 = 255.2 ng/mL - needs to be diluted.

after dilution = 53.0 ng/mL

OCT 40261-A = 2611.4 ng/mL- needs to be diluted

after dilution = 113.2 ng/mL

OCT 40262-A = 129.9 ng/mL

FRIDAY, 10/6/17

## Restriction Digest on Previously Isolated DNA Plasmid

1. Placed previously isolated DNA plasmid on ie (61 and 62).
2. Measured the concentration of the DNA Plasmids with the nanodrop (2µl).
    a. DNA plasmid 40261 : 356.2 ng/µl
    b. DNA plasmid 40262 : 300.5 ng/µl
3. Diluted DNA plasmid 1 : 3 to approximate 100 ng/µl and measured with nanodrop.
    a. DNA plasmid 40261 : 117.9 ng/µl
    b. DNA plasmid 40262 : 106.1 ng/µl
4. Labeled fresh 0.5 mL tubes 61 and 62.
5. For each tube, transferred 5 ul of 10x NEBuffer, 1 ul of StuI, 1 mL of cac 81, and 33 ul of dH2O.
6. Transferred 10µl DNA Plasmid 40261 to 0.5 mL tube 61 and 10µl of DNA plasmid 40262 to 0.5 mL tube 62.
7. Incubated 0.5 mL tubes at 37 degrees Celsius for fifteen minutes.
8. Added 10µl purple 6x loading dye to each of the 0.5 mL tube and mixed.
9. Gel was loaded as follows:

Table1

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | Cane | 1 | 2 | 3 | 4 | 5 | |
| 2 | 10µl of 2 log ladder | 10µl of digested ladder | 10µl of digested 40262 | 15µl of digested 40262 | 15µl of digested 40261 | 15µl of digested 40261 | 10µl of 2 lo ladder |

TUESDAY, 10/10/17

## Re-running Digested Plasmids on Gel Electrophoresis

1. Repeat of 10/6/17 procedure from Step 5
2. Placed unused gel in gel box for 30 min.
3. Pipetted 10µl in 1st and last lane (purple ladder).
4. Pipetted 15µl in 2nd and 2nd to last lane (3x concentration ol-digest).
5. Pipetted 30µl (double bond) in outer lanes.
6. Make ETBR solution and place gel in there and on SHAKER (5 min).
7. Image gel and results: Showed that plasmids were restricted at least once, but 100 bp fragmentts were not seen.
8. Conclusion: Restriction enzymes are to be checked for cut/not.

THURSDAY, 10/12/17

## Re-run Restrictions on 3X Concentrated Samples for 1 Hour

1. Labeled 0.5 mL tubes "61" and "62".
2. Transferred 5µl of 10x NEBuffer, 1µl of StuI, 1µl of Cac 81, and 33µl H2O.
3. Transferred 10µl of DNA plasmid 40261 to 0.5 mL tube "62".
4. Incubated tubes at 37 degrees Celsius for 1 hour.
5. Added 10µl 6x purple loading dye to each tube and mixed.
6. Gel was labeled as follows:

Table2

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | Lane | 1 | 2 | 3 | 4 | 5 | |
| 2 | empty | 10µl 2 log buffer | 15µl digested 40261 | 26µl digested 40261 | 26µl digested 40262 | 15µl digested 40262 | 10µl 2 log b |
| 3 | | | | | | | |

FRIDAY, 10/13/17

## Gel Extraction and PCR Purification

1. Weighed gel slices containing DNA fragment:
    a. 61a)0.3620g  b)0.3895g
    b. 62a) 0.2865g  b)0.3877g
2. Added gel solubilization buffer L3 in 3:1 ratio to gel in a 1.7 mL polypropylene tube.
3. Incubated tubes at 50 degrees Celsius for 15 min and inverted tubes every 3 min.
4. Incubated the tubes for an additional 5 min when gel slice appeared dissolved.
5. Pipetted and dissolved gel piece onto a column at 12000 x g for 1 minute. Discarded flow-through and placed column into wash tube.
6. Added 500µl wash buffer containing ethanol to the column. Centrifuged column at >12000 x g for one minute. DIscarded flow thorugh and placed column into wash tube. Centrifuged column at max speed for 2-3 min. Discarded flow-through.
7. Placed column into recovery tube. Added 50 mL elution buffer (E1) to the column. INcubated tube for a minute at room temperature. Centrifuged tube > 12000 x g/minute.
8. Stored, purified DNA at -20 degrees Celsius for immediate use.

SATURDAY, 10/14/17

## Purifying Gel Bonds from STUL-XBAL Digest

1. Weigh gel slices containing dna fragments:
    a. 61 > 0.2100 g
    b. 62 > 0.2230 g
2. Add Gel solubilization buffer L3 to excised gels in the tubes in a 3:1 ratop.
    a. 61 > 0. 63 g = 630µl
    b. 62 > 0.669 g = 669µl
3. Incubate at 50 degrees Celsius for 10 minutes ad invert tube every 3 minutes. After the gel slie appears dissolves, incubate tube for an additional 3 min.
4. Add 1 gel volume of isporopanol to dissolved gel slices. Mix well.
    a. 61 > 210µl
    b. 62 > 223µl
5. Pipet 850µl of dissolved gel pieces into wash tubes. Centrifuge for 10 seconds in minifuge. Transfer liquid from wash tube to new Eppendorfs in case of problems.
6. Centrifuge column 12,000 x g for 1 minute. Transfer flow through into the same Eppendorf tubes.
7. Add 500 micro-liters of Wash Buffer containing ethanol to column. Centrifuge column at 12,000 x g for 1 minute. Transfer flow through to same Eppendorf tubes and place column into Wash Tube. Centrifuge column at max speed for 2-3 minutes. Transfer flow through.
8. Place column into a Recovery Tube. Add 50 uL of Dilution Buffer (E1) to column. Incubate for 1 minute at room temp. Centrifuge at 12,000 x g for 1 minute.
9. Store elution tube (purified DNA) at 4 C for immediate use or -20 C for long-term storage.

## Amplification of Previously Isolated DNA Fragments

Fwd- 31.4 nmol + 500 uL= 62.8 umol

Rev- 23.3 nmol + 500 uL= 46.6 umol

Added 5 uL of PCR buffer, 1 uL of dNTPs, 1 uL Primer A, 1 uL Primer P, 10 uL DNA Template, 31 uL of water, and 1 uL of Taq Poly.

MONDAY, 10/16/17

## Verification of PCR Amplification from 10/14

1. Heat and pour gels.
2. Wait 45 minutes to cool and remove comb.
3. Take 10 uL of samples from 10/14 and add 2 uL of loading dye.
4. Load Ladder: 1, 2, S1, S2, D1, Ladder, D2, I1, I2, ID1, ID2, Ladder
5. Run gel at 100 volts for 30 minutes.
6. Put in ethimium bromide and put on rotator for 5 minutes.

## Digestion of 10/14's Samples with CAC8I

1. Put 2.3 uL of each of 10/14's 40261 and 40262 plasmids into separate tubes. Label tubes.
2. Transfer 5 uL of 10X NEB Cutsmart Buffer, 1 uL of CAC8I, 41.7 uL of dH20.
   *Tube for 40262 cracked, so we transferred to another tube.
3. Incubate tubes at 37 C for 1 hour.
4. Heat inactivate at 65 C for 20 minutes.

TUESDAY, 10/17/17

## Restrict Plasmid Fragments with CAC8I and Run Gel for Confirmation

1. Pipet 10 uL of I1, I2, 1, 2, and Exon 1 into five tubes.
2. Pipet 10 X NEB Buffer 5 uL into each tube.
3. Pipet 1 uL of CAC8I into each tube.
4. Pipet 1 uL of STUL into each tube.
5. Add water to each tube so that final is 50 uL.
6. Mix components by pipetting the reaction mixture up and down, or by flicking the reaction tube.
7. Spin down tubes in a microcentrifuge.
8. Incubate for 1 hour at 37 C.
9. Take 2 uL of 6X gel loading buffer and add it to 10 uL aliquots of each previously digested sample.
10. Run gel for all samples.
11. For the Exon 1 sample containing STU8I dilute entire sample (50 uL) with 6X purple loading dye (10 uL).
12. Load all samples into gel and add a 10W molecular weight ladder. (Gene Ruler 1 kb)
13. Heat inactivate the remainders of the previously digested samples at 65 C for 20 minutes.

*Tubes 1 and 2 are equal gel purified plasmids for 40261 and 40262 that had previously been digested with STUI and XBAI and PCR amplified with MP Taq Polymerase. I1 and I2 used invitrogen taq polymerase.

FRIDAY, 10/20/17

## Mini Prep AddGene HTT Plasmids From E.Coli

1. Prepared lysis buffer (calculated amount needed).
2. Poured 1.5 mL of the culture into a microcentrifuge tube.
3. Spun in microcentrifuge tube for 3 min at room temp. Placed in 4 degrees Celsius - store unused portion of the original culture at 4 degreees Celsuis.
4. Removed media w/ aspiration, leaving bacterial pellets as dry as possible.
5. Resuspended bacterial pellet in 200 mL of ice cole alkaline lysis solution 1 with vigorous vortexing (by hand).
6. Added 250µl of freshly prepared alkaline lysis solution II to each bacterial suspension. Closed the tube tightly and mixed the contents by inverting the tube rapidly five times. Did not vortex - place the tube on ice.

## Exon 1 Assembly

Followed the NEBuilder HiFi DNA Assembly Reaction Protocol

## Finalize Exon 1 Assembly

**Purpose: To add the prefix and suffix fragments to the addgene insert to complete exon 1 assembly.**

**Materials:**
NEB Hifi Assembly Kit
Addgene Insert with adapter overhang (previously PCRed)
IDT exon 1 flanks (previously restricted)
Water

Table4

| | A | B | C |
|---|---|---|---|
| 1 | | **Concentration** | **Volume** |
| 2 | **Prefix Fragment** | | |
| 3 | Suffix Fragment | | |
| 4 | Addgene Insert | | |
| 5 | **Total Volume of Fragments** | | |
| 6 | **Gibson Assembly Master Mix (2x) (µl)** | | |
| 7 | **Deionized Water (µl)** | | |
| 8 | **Total Volume (µl) ***** | | |

*Note: Experiment postponed bc previous digested exon flank was missing upon review of the digestion protocol it seems it was the restriction enzymes chosen were wrong. As StuI cuts exon flank in two places.

## G-Block Assembly

**Purpose: Begin Gibson Assembly so that we can PCR once primers are in.**

**Materials:**
IDT G-blocks (15)
NEB Hifi Builder Kit
Nuclease free water

Table5

| | A | B | C | D |
|---|---|---|---|---|
| 1 | **Concentration Range of DNA fragments** | 02 - .5 pmols* | .2 - 1.0 pmols* | 0 pmols |
| 2 | **Total Volume of Fragments (µl)** | | | 10 |
| 3 | **Gibson Assembly Master Mix (2x) (µl)** | 10 | 10 | 10 |
| 4 | **Deionized Water (µl)** | 10 | 10 | 0 |
| 5 | **Total Volume (µl) ***** | 20 | 20 | 20 |

1. Normalized concentrations of all G-blocks to 10 fmol per microliter.
2. Take 5 microliters of each G-block sample and put into a new tube of each paired G-block sample.
3. Then add 10 microliters of Gibson assembly master mix to each tube.
4. Incubate for 15 minutes at 50 C in the thermocycler.

MONDAY, 10/23/17

## Creating Exon 1 Fragment via PCR

**Purpose: To couple IDT flanking sequences to AddGene HTT sequences.**

**Materials**
See procedure for PCR specific reagents

**Template DNA:**

PCRed out and Adapted AddGene Sequence

Exon1_flank_XmnI-Adapter-Prefix

Exon1_flank-Cac8I-Adapter-Suffix

**Primers:**

HTT-Assembly-Fwd

HTT-Assembly-REV

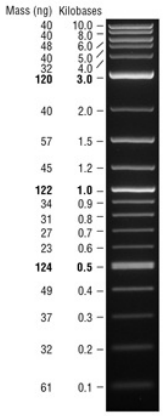
**Procedure:**
Follow protocol below

Flanking Region PCR Reaction

**Calculations:**
Previous gel of AddGene HTT sequences were compared to the NEB 2 log ladder.

- All amplified parts appeared to coorespond to approximately 100 ng on gel.  Gel lanes were loaded with 10ul of sample, thus 10 ul of each sample = ~100ng.

image.png




Flanking region DNA total = 1 pg in 50 ul

- Therefore, 5 ul = 100 ng

Primer Dilution:

- Starting quantity = 35 nM diluted into 100 ul
    - 350 uM/L = stock primer concentration
- 1 ul of stock primer into 34 ul of H2O = 10 uM
    - 1.25 ul of diluted stock = 0.5uM when diluted into a 25ul total reaction volume.

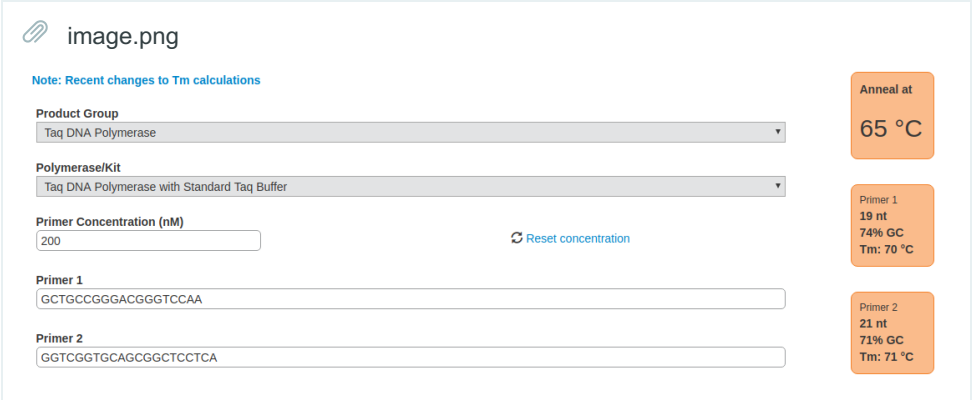
dNTP calculations

- 10 mM starting Conc.
    - dilute 1:2 to get 5mM

Taq Pol Calculations

- 5 U/uL starting Conc.
    - dilute 1:10 to get 0.5 U/uL

Anneling tempature calculations:




Elongation Calculations (1min / 1Kb)

- Size of AddGene insert + AddGene Primer overlap =

**Records of Quantities Used:**

Table6

| | A | B | C |
|---|---|---|---|
| 1 | Componet | Volume (uL) | Final Conc |
| 2 | AddGene HTT DNA | 9 | ~ 90 ng |
| 3 | cut IDT Flaking DNA | 9 | 90 ng |
| 4 | 10x PCR buffer | 2.5 | 1 X |
| 5 | 5mM dNTP Mix | 1 | 0.2 mM (each) |
| 6 | 10 uM forward primer | 1.25 | 0.5 uM |
| 7 | 10 uM reverse primer | 1.25 | 0.5 uM |
| 8 | 0.5 U/uL Taq Polymerase * | 1 | 2.5 U / rxn |
| 9 | Water | 0 | n/a |

***Add *Taq*Pol after intial denatuartion at 95˚C for 10 min**

**PCR Protocol As Implemented:**

1. Follow protocol Flanking Region PCR Reaction
- Cycle Name: 95C-10M

4. Store DNA at -20˚C

Post Hot start cycle values

| | A | B | C |
|---|---|---|---|
| 1 | Tempature | Time | Cycle name |
| 2 | 95˚C | 45 s | Melt |
| 3 | Tm - 5˚C (https://tmcalculator.neb.com/) | 45 s | Anneal |
| 4 | 72˚C | 1 min / kb | Elongate |

- Cycle Name: JHF

3. End cycle as follows

Final PCR steps

| | A | B | C |
|---|---|---|---|
| 1 | 72˚C | 10 min | Final Elongation |
| 2 | 4˚C | Indefinitly | Hold Chilled |

- Cycle Name: 95C-10M

4. Store DNA at -20˚C

Expected Product (with varied CAG repeats):


TUESDAY, 10/24/17

## G-Block: 5-Way and Sub Assembly

**Purpose: To subassemble paired G-blocks (Blocks 1-3, 4-5, 6-7, 8-9, 10-11, 12-13, 14-15) from the Friday's lab.**
To attempt a 5 way assembly of parts 1-5, 6-10, 11-15

**Materials (old sample name | new sample name):**
IDT G-blocks (15 in total: GB1-GB15)
Previously paird G-Blocks (pGBs)

- GB1-GB3 | pGB13
- GB4-GB5 | pGB45
- GB6-GB7 | pGB67
- GB8-GB9 | pGB89
- GB10-GB11 | pGB1011
- GB12-GB13 | pGB1213

- GB14-GB15 | pGB1415

NEB Hifi Builder Kit
Nuclease free water

Attempts were made to follow the NEBuilder HiFi DNA Assembly Reaction Protocol as closely as possible

Couple previously paired Gblocks as follows:

Pairing Coupled G-Blocks

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | Reaction Tube Name | paired G-Blocks Used | paird G-Block volume (µL) | numbe of paired G-Blocks | additional original G-Block added (µL) | number of original G-Blocks | Total vol added by DNA (µL) |
| 2 | cGB15 | pGB13, pGB45 | 10 | 2 | 2 | 5 | |
| 3 | cGB69 | pGB67, pGB89 | 10 | 2 | 2 | 4 | |
| 4 | cGB1015 | pGB1011, pGB1213, pGB1415 | 10 | 2 | 2 | 6 | |

* Justification: previous reaction should have correct concentrations of salts, and should still contain active polymerase and ligase. Thus this addition only adds exonuclease.

5-Way GBlock Assembly Setup

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | Reaction Tube Name | paired G-Blocks Used | paird G-Block volume (µL) | numbe of paired G-Blocks | Total vol added by DNA (µL) | Amount of 2x Assembly Buffer needed (µL) | * Amount of Assembly b used (µL) |
| 2 | 5-15 | GB1 - GB 5 | 5 | 5 | 25 | 25 | |
| 3 | 5-610 | GB6 - GB10 | 5 | 5 | 25 | 25 | |
| 4 | 5-1115 | GB11 - GB 15 | 5 | 5 | 25 | 25 | |

1. Incubate for 15 minutes at 50 C in the thermocycler.
2. Store at -20 C.

## Visualization of PCR Exon 1 Fragments

Purpose: To run a gel electrophoresis to visualize the PCR amplicons of the full Exon 1 Assembly

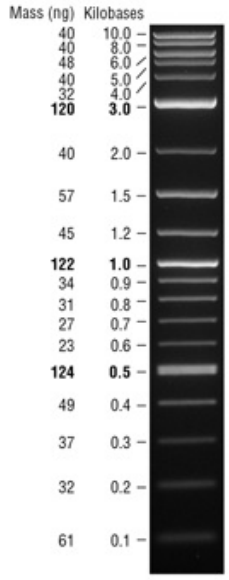
1. Split 10 lanes from precast 2% agarose gel.
2. Transfer 10ul from PCR tube to reaction tube.
3. Add 6x purple loading dye to each sample (2ul).
4. Load 2% gel as follows:

Table8

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | **Lane #** | 1 | 2 | 3 | 4 | 5 | |
| 2 | **Sample** | 2 log ladder | 1: MP Taq 24061 | 2: MP Taq 24061 | 3: Sat 24061 | 4: Invitrogen 24062 | J1: MP Tac 24061 |

2 log ladder will resolve as follows:

image.png




4. Run gel at 100 volts for 30 min.
5. Stain with ETBR with GENTLE shaking.
6. Image using gel imager.

WEDNESDAY, 10/25/17

## PCR Amplification of AddGene Plasmid Part Restriction

**Purpose:**
To take the HTT parts that Esther restricted out of AddGene plasmid samples 20461 and 20462 (from previous MiniPrep) and re-amplify out the HTT sequences now that we are sure about the cut sites (It was discovered last night that EcoRI not Cac8I should of been used).

**Procedure:**
Followed the Q5 Hi Fi 2X Master Mix Protocol for 50uL reaction

1. We labeled two PCR tubes '1' and '2' for the respective plasmid they were to soon contain.
2. Stock primers were diluted to a concentration of 10 µM into new labeled corresponding tubs as per calculated below.
3. We set up the thermocycler using a 10s denaturation, 20s annealing at 72°C , and a 20s elongation time.
4. Samples were loaded, and the cycle started.
5. 10ul was taken from each sample for gel loading.
6. Finished Samples (Labeled 1↑ and 2↑) were placed at -20°C.

**Calculations**

Stock primer concentrations were as follows: Fwd 62 µM  and Rev 46 µM.

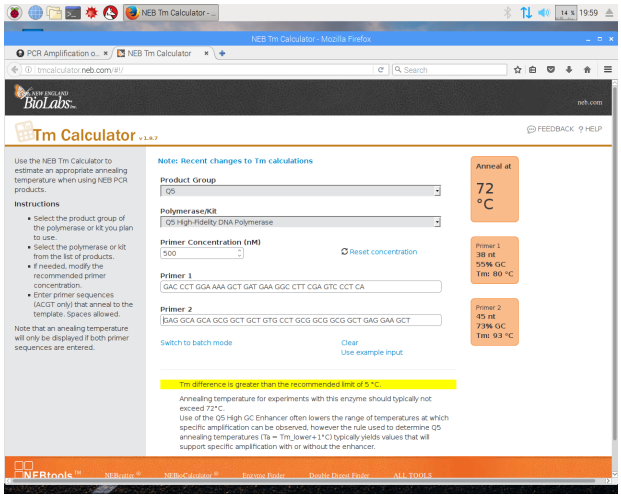
- AddGene_Fwd needs to be diluted at a 1:6.2 ratio.
    - 25 ul of stock was added to 130 ul nuclease free water.
        - (6.2*25 - 25 = 130)
- AddGene_Rev needs to be diluted at a 1:4.6 ratio.
    - 25 ul of stock was added to 115* ul nuclease free water.
        - (4.6*25 - 25 = 90)
        - * this was a mistake, actual concentration used is: 7.8mM.
            - 5.4ul stock was added to the working solutioncorrect this issue.

HTT AddGene Primers TMs were calculated on NEB's website using the **NEB $T_m$ Calculator**

(FWD): GAC CCT GGA AAA GCT GAT GAA GGC CTT CGA GTC CCT CA

(REV): GAG GCA GCA GCG GCT GCT GTG CCT GCG GCG GCG GCT GAG GAA GCT

2017-10-29-195922_1280x1024_scrot.png




**Results:**

1st gel had no ladder (loading dye was used by accident)

2nd gel center lanes didn't resolve well possibly due to incomplete curing of the gel

3rd gel was run with remaining PCR samples but did not contain enough DNA to be visualized

NEW PCR WILL HAVE TO BE RUN

## Part Assembly with EcoR1 Restriction Sites

**Purpose:**

Reassemble and cut existing Exon 1 parts such that there is a definite Eco R1 restriction site so that they can be added into a pSB1C3 backbone

1. Placed previously isolated DNA plasmid on ie (61 and 62)
2. Measured the concentration of the DNA Plasmids with the nanodrop (2µl).
    a. DNA plasmid 40261 : 356.2 ng/µl
    b. DNA plasmid 40262 : 300.5 ng/µl
3. Diluted DNA plasmid 1 : 3 to approximate 100 ng/µl and measured with nanodrop.
    a. DNA plasmid 40261 : 117.9 ng/µl
    b. DNA plasmid 40262 : 106.1 ng/µl
4. Labeled fresh 0.5 mL tubes 61 and 62
5. For each tube, transferred 4 ul of 10x NEBuffer, 1 ul of Eco R1, 1 ul of Xmn 1, and 25.6 ul of dH2O*
6. Transferred 10µl DNA Plasmid 40261 to 0.5 mL tube 61 and 10µl of DNA plasmid 40262 to 0.5 mL tube 62.
7. Incubated 0.5 mL tubes at 37 degrees Celsius for fifteen minutes.

8. Added 10µl purple 6x loading dye to each of the 0.5 mL tube and mixed.
9. Gel was loaded as follows:

Table10

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | Cane | 1 | 2 | 3 | 4 | 5 | |
| 2 | 10µl of 2 log ladder | 10µl of digested ladder | 10µl of digested 40262 | 15µl of digested 40262 | 15µl of digested 40261 | 15µl of digested 40261 | 10µl of 2 lo ladder |

*NOTE: NEBuffer and H2O was accidentally pippeted into the DNA Plasmids (tubes 61 and 62)
- So 18 ul of each 61 and 62 were pipetted into corresponding 61 and 62 tubes (the other ones)

THURSDAY, 10/26/17

## PCR Assembly with Alternating Paired Parts

**Purpose:**
The gibson reaction showed no bands, this is likely due to the low DNA concentrations used, thus we MUST PCR amplify.
As we don't have appropriate primers, we will have to try using G-Blocks as primers.

**Layout:**

**Part amplification:**
Just use the the GBs that should be flanking either side fo the assembly for primers (subistutue in FWD 1 and Rev 15 though)

**Full assembly:**
As we have a FWD primer for GB1 and a REV primer for GB15 we can use those.
Since GBs are double stranded, they act as forward primers for the GB after them and reverse primers for the GB preceeding them.
Thus we should only need even numbered GB as primers.

**Part Amplification Parameters:**

Table11

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | Tube Name | Part to amplify | Volume of Part used (µL) | Fwd Primer used | Volume Fwd primer (µL) | Rev Primer used | Volume R primer (µl |
| 2 | ↑ C1 | cGB15 | 4 | F2 (GB1-FWD) | 1 | GB5 | |
| 3 | ↑ C2 | cGB69 | 5 | GB6 | 10 | GB9 | |
| 4 | ↑ C3 | cGB1015 | 4 | GB10 | 20 | R9 (GB15-REV) | |
| 5 | ↑ 5-A | 5-15 | 4 | F2 (GB1-FWD) | 1 | GB5 | |
| 6 | ↑ 5-B | 5-610 | 5 | GB6 | 10 | GB10 | |
| 7 | ↑ 5-C | 5-1115 | 4 | GB11 | 20 | R9 (GB15-REV) | |

**Full Assembly Parameters:**

Table12

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| 1 | **Tube Label** | **Assembly type** | **Volume of Part used (µL)** | **Even GB used (µL)** | **volume2F 9R primer (µL)** | **Amount of Q5 master mix (µL)** |
| 2 | All cGB | Coupeled -Paired | 1 (3x) | 6.5 (x7) | 1 (2x) | 50 |
| 3 | All 5GB | 5 way | 1 (3x) | 6.5 (x7) | 1 (2x) | 50 |

**PCR Cycler settings used**

Table13

| | A | B | C |
|---|---|---|---|
| 1 | **STEP** | **TEMP** | **TIME** |
| 2 | Initial Denaturation | 98°C | 30 seconds |
| 3 | 28 Cycles | 98°C | 10 seconds |
| 4 | | 70°C | 25 seconds |
| 5 | | 72°C | 4.5 min |
| 6 | Final Extension | 72°C | 5 minutes |
| 7 | Hold | 4°C | |

FRIDAY, 10/27/17

## PCR of 5-way Assembly with Correct Primers

**Purpose:**
to try and get a cleaner PCR using correct primers

**Procedure:**
We will be following Q5 Hi Fi 2X Master Mix Protocol

SATURDAY, 10/28/17

## Gel Verification of PCR Primer-Adaption of Exon 1 - Part 1

# Purpose

To verify PCR reaction of Primer-Adaption to Exon 1 through gel electrophoresis

# Procedure

1. Mixed 10 uL of sample and 2 uL of loading dye into a microcentrifuge tube.
2. Loaded samples into 2% agarose gel as follows.

Table14

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | **Lane** | 1 | 2 | 3 | 4 | 5 | |
| 2 | **Sample** | 2 Log Ladder | 40261 | 40262 | Saturday 40261 | Invetrogen 40262 | 2 Log Ladd |

3. Ran gel for 30 minutes at 100 volts.
4. Stained with ethimium bromide as per protocol Ethidum Bromide DNA Gel Staining

SUNDAY, 10/29/17

## PCR Amplification of AddGene Plasmid Part Restriction

**Purpose:**
To take the HTT parts restricted out of AddGene plasmid samples 20461 and 20462 (from previous MiniPrep) and re-amplify out the HTT sequences now that we are sure about the cut sites (It was discovered last night that EcoRI not Cac8I should of been used).

pEGFP-Q23 (AddGene Plasmid #40261)

pEGFP-Q74 (AddGene Plasmid #40262)

**Desired Outcome:**
Clean bands at ~ 450 MW

**Procedure**
We followed the Q5 Hi Fi 2X Master Mix Protocol for 50uL reaction:

1. We labeled two PCR tubes '1' and '2' for the respective plasmid they were to soon contain.
2. Stock primers were diluted to a concentration of 10 µM into new labeled corresponding tubs as per calculated below.
3. We set up the thermocycler using a 10s denaturation, 20s annealing at 72°C , and a 20s elongation time.
4. Samples were loaded, and the cycle started.
5. 10ul was taken from each sample for gel loading.
6. Finished Samples (Labeled 1↑ and 2↑) were placed at -20°C.

**Calculations**

**Primer Dilutions (previously done)**
Stock primer concentrations were as follows: Fwd 62 µM and Rev 46 µM.

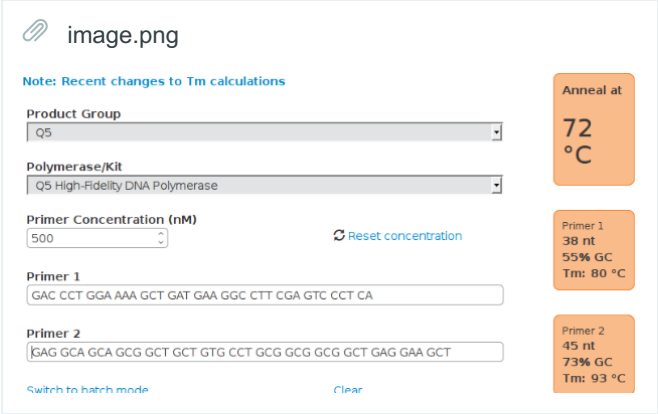
- AddGene_Fwd needs to be diluted at a 1:6.2 ratio
    - 25 ul of stock was added to 130 ul nuclease free water
        - (6.2*25 - 25 = 130)
- AddGene_Rev needs to be diluted at a 1:4.6 ratio
    - 25 ul of stock was added to 115* ul nuclease free water
        - (4.6*25 - 25 = 90)
        - * this was a mistake, actual concentration used is: 7.8mM
            - 5.4ul stock was added to the working solutioncorrect this issue

**Primer TMs**

HTT AddGene Primers TMs were calculated on NEB's website using the **NEB $T_m$ Calculator**

(FWD): GAC CCT GGA AAA GCT GAT GAA GGC CTT CGA GTC CCT CA

(REV): GAG GCA GCA GCG GCT GCT GTG CCT GCG GCG GCG GCT GAG GAA GCT




**Results:**

See Results from Gel Test: Gel Verification of PCR Primer-Adaption of Exon 1 - Pt2

Gel was run and bands at expected MWs were seen (and cut out)

Band of unmodified template were also seen (~350MW)

Small 200 MW products were also seen, but hard to determine where they came from.

Smearing (likely due to overamplification) was also seen

**Conclusions**

Extracted bands should be fine to work with, however DNA concentrations might be a bit low, thus a second set of bands should be extracted from another gel

## Gel Verification of PCR Primer-Adaption of Exon 1 - Part 2

**Purpose:**

To verify PCR Amplification of AddGene Plasmid Part Restriction Pt2 using gel electrophoresis

**Procedure**

1. Mixed 10 uL of sample and 2 uL of loading dye into a microcentrifuge tube.
2. Chose NEB 2 log ladder.
3. Loaded samples into 2% agarose gel as follows.

Table16

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | **Lane** | 1 | 2 | 3 | 4 | 5 | |
| 2 | **Sample** | 2 Log Ladder-blue | 40261-blue | 40262-blue | Saturday 40261-blue | Invetrogen 40262-blue | 40261-blac |

3. Ran gel for 33 minutes at 100 volts.
4. Stained with ethimium bromide as per protocol Ethidum Bromide DNA Gel Staining

# Results

image.png


Bands between 400 and 500 look good!

MONDAY, 10/30/17

## HTT Exon 1 UTR Build

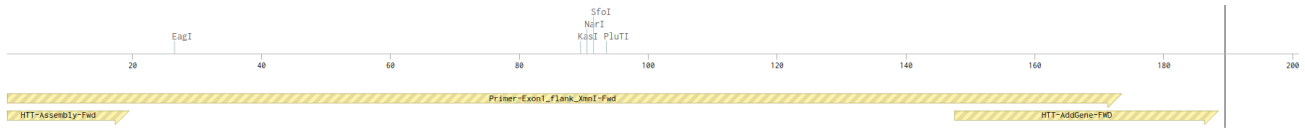
# Simplification

As the only difference between pEGFP-Q23 (AddGene Plasmid #40261) and pEGFP-Q74 (AddGene Plasmid #40262) is the CAG (poly Q) repeat site, for simplicity we will stck with pEGFP-Q23 (AddGene Plasmid #40261) , or from now on to be known at Q23.

# AddGene Exon 1 Pieces

Here is the 5' UTR HTT Exon 1 pieces as shipped by IDT

image.png




Here are the  3' End adaptors (due to high GC content):

image.png


These can be see how these align with the full gene here:

Full Assmbly: Wild Type Huntinton mRNA - GBlock Aligned

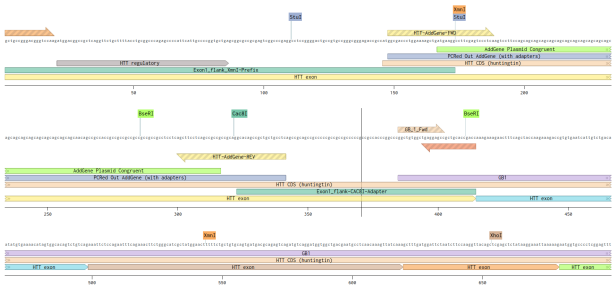
- As one can see the XmnI / StuI cut site is located perfectly for the 5' UTR overlap assembly
- The 3' Cut site corresponds to an Cac8I site that also exists in the HTT gene.

# Q23 AddGene Insert

Congruency (lime green) between Exon 1 and The AddGene Q23 insert

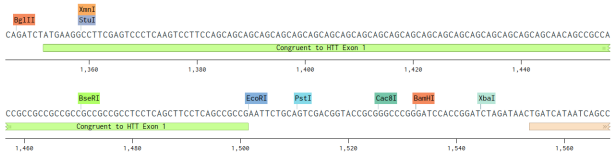
Aligned with NM_002111 looks like:

image.png



Aligned with pEGFP-Q23 (AddGene Plasmid #40261) looks like:

image.png




# Q23 Restriction sites

pEGFP-Q23 (AddGene Plasmid #40261) has **34 cut sites for Cac8I** but only one for XmnI, EcoRI, PstI, BamHI and XbaI. StuI has 2!

StuI can be substituted for XbaI.

Cac8I cuts 203 bases upstream of the XmnI / StuI cut site

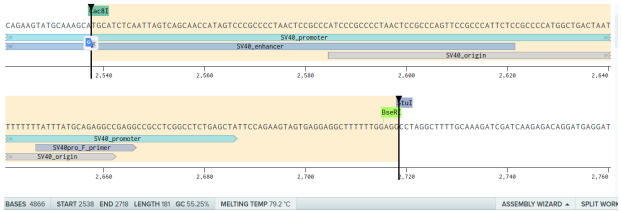
image.png

Cac8I cuts 167 bases down stream of the  XmnI / StuI cut site

image.png

These sites are hard to separate on gel.  Further, many other Cac8I sites cut similar length digests.  In particular upstream of the second StuI site (flanking our sequence of interest there is a 181 base cut:

image.png



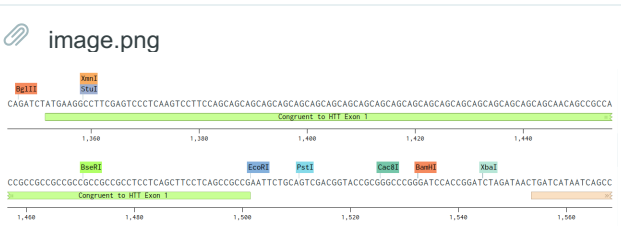
Thus, it's not likely that the reduction of cut sites by serial digestion after purification of the smaller StuI fragment will help much. Thus cutting with Cac8I doesnt seem like a good idea (and indeed we did not see the fragment we hoped for when we did cut there.

Thus a better secondary cutter would be EcoRI or XbaI.

# Q23 Fragments

Possible fragments when attempting to remove HTT exon 1 from pEGFP-Q23 (AddGene Plasmid #40261) are the following:

image.png



XmnI - EcoRI

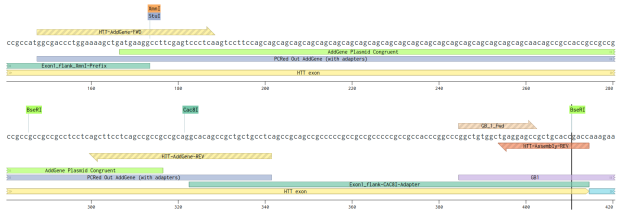
XmnI - XbaI

etc.

However, when PCRing out the gene isn't really doable because the primer is designed with a flanking region.  Thus **EcoRI should be used.**

The remaining insert (dark blue) after PCR is:

image.png



## Restricting the Mini Gene

**Isothermal assembly:**
The miniGene and the adapted AddGene plasmid can be coupled and amplified via Gibson Assembly

- primers for this seem to have no adapters so they truncate at the ends of the miniGene

From here we can gibson assemble (or even possibly PCR in) the remaining piece.

Based on the piece we assembled it seems I ordered and adapter for ,
This part is not supplied in 2017 (or ever) - mistake was made becasue I searched igem for pSB1C3 and this was the first thing that turned up (I didn't realize there are some parts that dont' follow the BBa prefix notation).