





- Sample yang digunakan adalah DNA
 Metode ekstraksi DNA.
- Gold standard locus:
 - · COX1 (hewan),
 - rbcL dan matK (tumbuhan),
 - ITS (jamur),
 - 16s RNA (bakteri)

Design primer

- Tidak seluruh sekuen digunakan hanya sekuen "representatif".
- Disesuaikan dengan kebutuhan analisis/ identifikasi: level spesies, genus, famili, dst.

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Gold standard daerah target untuk barcoding diantaranya:

- 1. Hewan: COX1 (cytochrome c oxidase 1)
- 2. Tumbuhan: *rbcL* (large subunit RuBisCo), *matK* (maturase K)
- 3. Fungi: ITS
- 4. Prokariot: 16S rRNA (ribosomal RNA subunit)

Slow evolving region: useful for distantly related taxa (genus, family, ordo)

Fast evolving region: needed when identifying more closely related species such as cryptic/sibling species groups.

PENGUMPULAN SEKUEN GEN TARGET

 Lakukan langkah yang sama untuk spesies nyamuk lainnya dan simpan sekuen fasta gen COX1 pada "notepad".

Berikut beberapa complete sequences COX1 gene:

- Aedes aegypti: NC_035159 (location: 1298-2834)
- Aedes albopictus: NC_006817 (location: 1436-2972)
- Culex pipiens pallens: NC_015079 (location: 1446-2982)
- Culex quinquefasciatus: NC_014574 (location: 1446-2982)
- Anopheles cruzii: NC_04464 (location: 1445-2983)
- Anopheles gambiae: NC_002084 (location: 1424-2960)

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- Data sekuen gen target dapat dicari dan diunduh dari website NCBI (https://www.ncbi.nlm.nih.gov/).
 - Misal, anda diminta merancang primer gen cytochrome c oxidase 1 (COX1) dari beberapa spesies nyamuk, yaitu: Aedes, Culex, dan Anopheles.

	NIH National	Library of Medicine			
Lakukan pencarian complete genome pada NCBI	Nec	ectide v Aedes			o South
 Pilih "<u>nucleotide</u>" dari menu drop down 					
	NCBI Home	Welcome to NCBI			Popular Resources
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			3-8-6	6	New Available/ Updated NCBI Datasets Command-Line Tools

PENGUMPULAN SEKUEN GEN TARGET

Untuk merancang primer barkoding, gunakan complete sequence dari gen target.

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Lakukan penyaringan data dengan memilih menu yang ditunjuk oleh panah.

• RefSeq (dari menu source databases) dan

• Mitochondrion (dari menu genetic compartments)

Pada bagian kanan halaman hasil (kotak merah),

- Terdapat informasi mengenai spesies Aedes yang data complete genome dari gen COX1 sudah tersedia di NCBI.
- Unduh data complete sequence gen COX1 dari beberapa spesies Aedes, misal: A. aegypti dan A. albopictus



PENGUMPULAN SEKUEN GEN TARGET

• Pilih salah satu hasil pencarian setelah disaring, misal "Aedes aegypti" (panah merah)

- Kemudian gunakan shortcut find (<u>Ctrl+F</u>) untuk mencari "COX1" (panah kuning),
- kemudian klik tautan genelD untuk gen COX1 (panah hijau).



- Anda akan diarahkan ke halaman genome viewer yang menunjukkan lokasi COX1 pada genome nyamuk Aedes aegypti.
- Buka tautan FASTA pada tab baru (panah),
- Gambar kanan menunjukkan halaman FASTA seq COX1 organisme Aeded aegypti



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PENGUMPULAN SEKUEN GEN TARGET

Tugas UAS (bagian 1):

- Cari dan tentukan kelompok organisme yang akan anda identifikasi menggunakan Teknik DNA Barkoding.
- Cari dan tentukan gen target yang sesuai untuk mengindentifikasi kelompok organisme tersebut.
- Kumpulkan sekuen gen target minimal dari 10 organisme yang ingin anda identifikasi.
- Simpan file dalam notepad.



2. PENJAJARAN SEKUENS DAN REKONSTRUKSI POHON FILOGENETIK



UNIVERSAL PRIMER DESIGN

- Primer can be designed to amplify specific regions from specific organism DNA.
- Primer can also be designed to amplify **specific regions from various organisms**. Strategy:
 - Align groups of sequences targeted for amplification
 - Find the most conservative region at 5' and 3' ends
 - Design forward primer at the 5' conservative region
 - Design reverse primer at the 3' conservation region
 - Perform primer pair "Quality Control" to find the best pair
 - Ensure uniqueness in all template sequence
 - Ensure uniqueness in possible contaminant sources

LANGKAH MELAKUKAN PENJAJARAN MENGGUNAKAN SOFTWARE MEGA

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REKONSTRUKSI POHON FILOGENETIKA DENGAN MEGA SOFTWARE

Buka aplikasi MEGA X, Lalu pilih, menu **phylogeny**, kemudian pilih **construct/test Neighbor-Joining Tree**













• Penjajaran sekuen (*alignment*) dilakukan untuk mengidentifikasi daerah lestari (*conserved region*) dan daerah polimorfik (*polymorphic region*).

- Daerah lestari gen: memiliki sekuen DNA yang sama pada tingkatan taksonomi yang berbeda
- Daerah polimorfik gen: daerah yang memiliki variasi sekuen DNA pada tingkatan taksonomi yang berbeda.
- Primer di desain pada daerah yang lestari sehingga sepasang primer dapat dipergunakan untuk **mengamplifikasi DNA** dari beberapa organisme.
- Urutan daerah polimorfik digunakan untuk mengidentifikasi organisme.

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Lakukan alignment/penjajaran pada sekuen gen COX1 yang sudah dikumpulkan, menggunakan tools clustalW pada software MEGA, **BioEdit**, atau **MUSCLE** (web-based tools).

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Ins PC	Mangifera_matK_seq	NC 015075 Cu 7CGCGACAATGAC	ProMi, Protein Maximum Likelihood program	GCTGGAA7AGT7GGAACTTCTTTAAGT77AC7AAT7CGAGCAGAATTAAGTCAACCAGG7GTA7
	Mangifera_rbcL_seq	NC_0286161Cu 7CGCGACAATGAC	Proteist> Hitch phylogenetic tree	SCTSGAA7AGTAGGTACTTCTTTAAGTATTT7AAF7CGAGCAGAATTAAGTCAACCTGGAGTAT
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PENJAJARAN (ALIGNMENT)

Lakukan alignment/penjajaran pada sekuen gen COX1 yang sudah dikumpulkan, menggunakan tools clustalW pada software MEGA, BioEdit, atau **MUSCLE** (web-based tools).

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PENJAJARAN SEKUEN

Tugas UAS (bagian 2):

Lakukan penjajaran sekuen gen target pilihan anda menggunakan:

- 1. Offline tools (installation needed): Mega, BioEdit
- 2. Web based tools provided by ebi.ac uk
 - MUSCLE: https://www.ebi.ac.uk/Tools/msa/muscle/
 - Clustal omega: <u>https://www.ebi.ac.uk/Tools/msa/clustalo/</u>







Primer adalah urutan sekuen nukleotida pendek (18-30 basa) yang biasanya Digunakan untuk amplifikasi sekuen DNA spesifik secara *in vitro* pada proses *polymerase chain reaction* (PCR)

Desain Primer dapat dilakukan dengan menggunakan software bioinformatik. Bisa menggunakan software berbasis web atau pun perangkat lunak yang di install pada komputer

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TAHAP DESAIN PRIMER

- 1. Menentukan dan mencari sekuen DNA target: gen, intergenic, ekson, intron, UTR
- 2. Penjajaran (alignment) sekuen DNA
- 3. Menentukan daerah DNA yang akan diamplikasi: polymorphic region
- 4. Uji kualitas primer yang telah didesain
- 5. Uji spesifisitas primer yang telah di desain (in silico PCR)



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SOFTWARE DESAIN PRIMER

Software berbasis web yang dapat digunakan untuk desain primer

- Primer-Blast (NCBI)
- Primer3
- GenScript
- PerlPrimer
- RExPrimer

Software pada personal komputer yang digunakan untuk desain primer

- PrimerSelect
- PrimerPremier6
- Fast PCR
- PrimerDesign
- Oligo 6
- Geneious

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WEBPAGE DESAIN PRIMER

- Pencarian data sekuen gen target: • NCBI: https://www.ncbi.nlm.nih.gov/
- Desain primer:
 - NCBI primer BLAST: https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome
 - blast/index.cgi?LINK_LOC=BlastHome
 Primer3 Plus: <u>https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi</u>
- Quality check kandidat primer:

 - OligoAnalyzer: <u>https://sg.idtdna.com/</u>
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- NCBI primer BLAST



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ANALISIS KUALITAS PRIMER MENGGUNAKAN OLYGOANALYZER

1. Akses website (<u>https://sg.idtdna.com/</u>).

Pada bagian paling bawah website klik OligoAnalyzer Tool



ANALISIS KUALITAS PRIMER MENGGUNAKAN OLYGOANALYZER

- Lakukanlah sign in jika sudah memiliki akun, namun jika belum lakukan register terlebih dahulu kemudian sign in
- 3. Pilihlah salah satu primer forward dari kandidat primer hasil pick primer di Primer3, kemudian *paste* sekuen primer tersebut di OlygoAnalyzer. Lalu pilih *analyze*. Lakukan hal yang sama untuk mengetahui kualitas primer reverse

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ANALISIS KUALITAS PRIMER MENGGUNAKAN OLYGOANALYZER

4. Setelah pilih analyze, OlygoAnalyzer akan menampilkan rincian dari primer yang dianalisis, seperti: panjang sekuen primer, suhu melting, suhu annealing dan sebagainya. Untuk mengetahui struktur sekunder internal primer, klik bagian self-dimer dan hairpin

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ANALISIS KUALITAS PRIMER MENGGUNAKAN BEACON DESIGNER

- · Buka website Beacon Designer. Isikan identitas primer.
- Pada drop-down [Type], pilih "Primer pair". Salin urutan primer forward dan reverse pada kotak tersedia. Klik [Analyze]

Beacon Free Designer Edition		PREMIER Biosoft	
Cligo Analysis Sequence Analysis			Beacon Designer Full Versio
Assay Type:			
	ItaqMan [®] ○ SYBR [®] Green		
Name:	NC_029243jA sinensis_rpoC1	[Optional]	
Description	\	[Optional]	
Type:	Primer pair v 2		
Serve Primer	8 GTGTGGTATTTGAAACGTCTTCCT	24 bp	
Azā-sense Primer		3 23 tp	
Prote:	5 X	bp	
Reaction Conditions:			
Nucleic Acid Concentration:	0.25 ///		
Nono Ion Concentration	50 mM		
Free Ma++ Concentration	5 mM		
Total Na+ Concentration:	332.84 mM		
		4	

ANALISIS KUALITAS PRIMER MENGGUNAKAN BEACON DESIGNER

Anary trajector Manary trajector Manary trajector Manary trajector Anary trajector Manary trajector <th colspa<="" th=""><th>Name: NC_029243 A.sinensis_</th><th>rpoC1</th><th></th><th></th><th></th><th></th><th></th><th></th><th>November 10, 2021</th><th></th></th>	<th>Name: NC_029243 A.sinensis_</th> <th>rpoC1</th> <th></th> <th></th> <th></th> <th></th> <th></th> <th></th> <th>November 10, 2021</th> <th></th>	Name: NC_029243 A.sinensis_	rpoC1							November 10, 2021	
Reaction containing Operation of the left of the l	Description:								Assay Type: TagMan®	1	
Nutlet Act Greeneration (M) 0.28 Measured Concentration (M) 50 Free Marcinet (M) 5 Test Marcinet (M) 50 32.84 Seese Privace OUTODALTANALACUETOR (M) 0 32.84 32.84 Seese Privace OUTODALTANALACUETOR (M) 0 0.00 32.84 Seese Privace Test Marcinet (M) 0 0.00 32.84 Seese Privace OUTODALTANALACUETOR (M) 0 0 0 Length Tm (C) 0 0 0 0 24 SEA3 41.87 2 3.0 3.1 4.8 As ease Privace TOTODALTANALACUETOR 0 0 0 0.8 As ease Privace Totodal toto	Reaction Conditions:										
Pres Net* Concentration 5 Tate late - Concentration 333 at Sense Primer d10100XT104AC010TIGT 333 at 333 at 333 at Sense Primer d10100XT104AC010TIGT 5 66% 0 C tamp Sense Primer CTCID/C000CH0A/M0178 30 at 30 a	Nucleic Aci	d Concentration nM)	0.25			Monovalent Concentration (mM)			50		
States Official/TTAMACOUNTION Leggin Top OPEN OPEN OPEN OPEN OPEN Margin (Lag) Margin (Lag) <td>Free Ng+</td> <td>Concentration mM)</td> <td>5</td> <td></td> <td></td> <td>Total Na+ Concentration (mM)</td> <td></td> <td></td> <td>332.84</td> <td></td>	Free Ng+	Concentration mM)	5			Total Na+ Concentration (mM)			332.84		
$\begin{tabular}{ c c c c c c c c c c c c c c c c c c c$	Sense Primer:	GTGTGGTATTTGAA	ACGTCTTCCT								
24 54.3 41.97 2 -30 3.3 -68 And searce Planes CTCRECODDCISATION ($\frac{1}{2}, \frac{1}{2}, \frac$	Length (bp)	Tm (^A C)	GC%	GC Clar	ι ρ	Cross Dimer (ΔG)	Self Dir (4G)		Hairpin (ΔG)		
And texperiment CTICUICODOCIDATIONTION Lange Open Provided Open Provided	24	58.43	41.67	2		-2.0	-3.3		-0.6		
Length 23 Tm (s) (s) 0°C/s 0°C Camp Constraint Beffere (s) Beffe	Anti-sense Primer:	CTCTCATCCGGCTG	AAGTAGTTA							1	
23 611 47.01 2 -30 d.1 00 As a rule of fhumb: Never accest primers where the 3' end has 3 bp matches, as these will tend to form primer dimers professionally over hybridizing with the sequence. If self-dimers y cross dimers cannot be avoided, chose primers with AGs more negative finan - 0.5 kcalimol. Delta C -0.36 kcalimols AGs more negative than -0.5 kcalimol. one closest to zero). Discard primers with AGs more negative finan -0.5 kcalimol. 0	Length (bp)	Tm (^A C)	GC%	GC Clar	IP.	Cross Dimer (ΔG)	Self Dir (4G)		Hairpin (ΔG)		
As a rule of thumb: Never accept primers where the 3' end has 3 bp matches, as these will tend to form primer dimers preferentially over hybridizing with the sequence. If self-dimers or cross dimers cannot be avoided, hose primers with the highest -ΔG more negative than -3.5 kcal/mol. Beta G -6.14 kcal/mole Beta G -6.14 kcal/mole Beta G -6.05 kcal/mole Be	23	58.1	47.83	2		-2.0	43		0.0		
	As a rule of thumb: • Never accept primers primer dimers preferen • If self-dimers or cross −ΔG (meaning the leas ΔGs more negative that Reference: Thornton and Basi	where the 3' end has 3 bp tially over hybridizing with dimers cannot be avoided st negative number—the c an -3.5 kcal/mol. J, 2011 (https://doi.org/10.100	matches, as these the sequence. chose primers with ne closest to zero). 2/bmb.20461)	will tend to fo h the highest . Discard prim	irm iers with	Delta G -6.14 kcal/mole Base Pairs 3 S' GOSCTAACTICAATGICATCC III : :: : :: :: :: S' COLTAACTICAATGICATCCA	Delta G Base Pai C 5' : G 3' CC	-0.96 irs 2 CTACIGT	skcal/mole gggctaacticaatgtcaic : : aacticaatgggg	.cc	



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HASIL PRIMER-BLAST APABILA MENU 'ORGANISM' DIISI DENGAN SPESIES TARGET

PCR template none							
city of primers Targe	t templates were found in selected database: Nucleotic	de collection (nt) (Orga	anism limite	ed to Aquila	aria)		
Other reports Sea	ch Summary						
	A						
etalled primer repoi	15						
Delesson and a d							
Primer pair I							_
Forward primer	Sequence (5->3)	Length 24	50.40	41.67	6 00	2.00	
Reverse primer	CTCTCATCCGGCTCAAGTAGTTA	23	59.37	47.83	4.00	2.00	
Products on target temp >NC_052859.1 Aquilaria.	ubintegra chlomolast, complete genome						
- Ho_conserver Aquinana	autregra cirioropian, comprete genome						
product length - 90							
Forward primer 1	GTGTGGTATTTGAAACGTCTTCCT 24						
Template 24	60 24737						
Powonce primer 1	CICICALCOGCICALGIAGUA 22						
Template 239	23989						
>MN147870.1 Aquilaria s	inensis chloroplast, complete genome						
product length = 75	18						
Forward primer 1	GTGTGGTATTTGAAACGTCTTCCT 24						
Template 24	74 24751						
Bewence primer 1	CTUTCHTCCCCTCARCTAC 33						

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