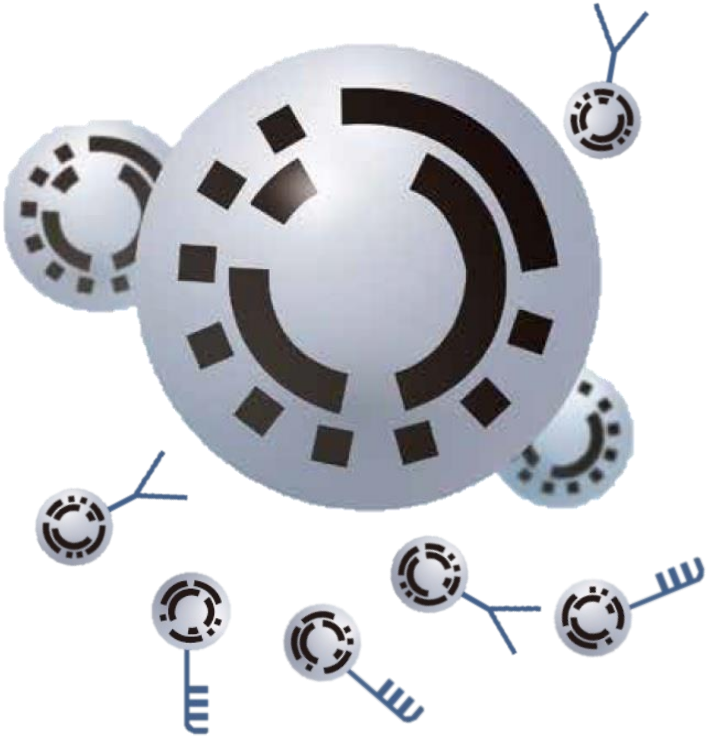
Molecular Application



Overview

- Introduction
- Material and Equipment
- General Workflow of Designing a Nucleic Acid Assay *
- Determine Nucleic Acid Assay Format *
- Primer Design
- PCR Condition
- Probe Design
- Hybridization Condition
- Troubleshooting

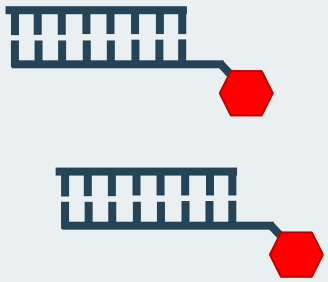
* The focus of following contents is on the design of PCR-/RT-PCR-based target amplification technologies and selected mutation enrichment strategies. PlexBio's πCode MicroDisc technology supports alternative target- or signal-amplification formats such as branched-DNA signal amplification.

Introduction

- PlexBio's πCode MicroDisc technology supports a broad range of applications for users to develop their own multiplex assays, including single nucleotide polymorphism (SNP) analysis, rearrangement detection, insertion/deletion detection and many more.
- Multiplex molecular assay is accomplished by the detection of fluorescence signal intensity detected on the different target-specific probe-amplicon complexes associated with unique πCode MicroDiscs.
- Biotin-labeled amplicons are hybridized to specific probes, which are coupled with unique πCode MicroDiscs, and then labelled fluorescently with streptavidin-phycoerythrin (SA-PE) conjugate.

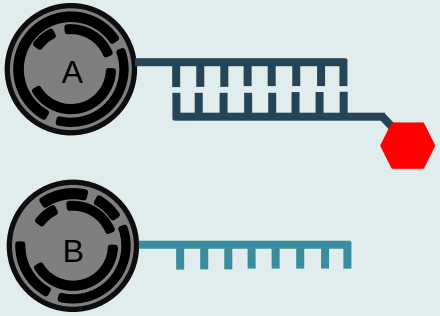
Introduction

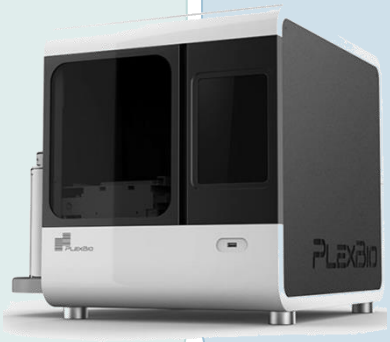
Amplification



DigiPlex™ Thermocycler

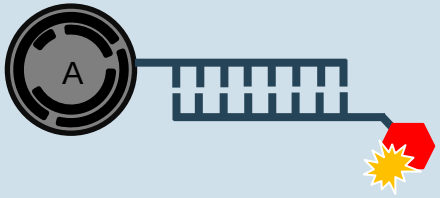
Hybridization



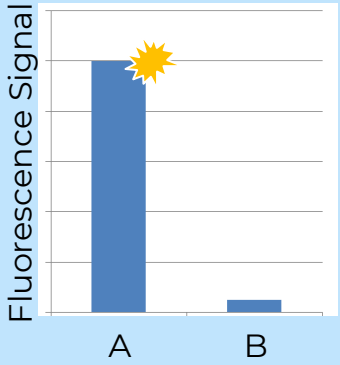


IntelliPlex™ 1000 πCode Processor

SA-PE Labeling



Detection



PlexBio™ 100 Analyzer

Material and Equipment

Reagent & Consumables	Vendor
πCode™ MicroDisc, Pre-activated	PlexBio, Cat No. 80131, 80132, 80133, 80134
πCode™ Coupling Buffer Set	PlexBio, Cat No. 80214
DNA Hybridization Buffer (Ready to use)	PlexBio, Cat No. 80206
10X Wash Buffer	PlexBio, Cat No. 80210
SA-PE Solution (Ready to use)	PlexBio, Cat No. 80203
PCR/RT-PCR primer sets for target amplification	IDT or other vendor *See Page. 12 for design detail
Appropriate PCR/ RT-PCR amplification system including enzymes and buffer	Any suitable brand
5' Amine modified probes	IDT or other vendor *See Page. 12 for design detail

Material and Equipment

Instrumentation & Accessories	Vendor
PlexBio™ 100 Analyzer	PlexBio, Cat No. 80000
Industrial Computer	PlexBio, Cat No. 80002
DigiPlex™ Thermocycler	PlexBio, Cat No. 80018 or equivalent
IntelliPlex™ 1000 πCode Processor	PlexBio, Cat No. 80033
Magnetic Stand (for 0.5mL)	PlexBio, Cat No. 80014
Rotator	PlexBio, Cat No. 80036
U Tray	PlexBio, Cat No. 80023
V Tray	PlexBio, Cat No. 80024
96-well Microplate (Flat)	PlexBio, Cat No. 80025
IntelliPlex™ Calibration Kit	PlexBio, Cat No. 80035-R

Material and Equipment

Instrumentation & Accessories	Vendor
0.2 mL PCR tubes	Any suitable brand
1.5 mL Nuclease-free microcentrifuge tubes	Any suitable brand
Calibrated micropipette	Any suitable brand
Disposable filter pipette tips (2-1000 µL)	Any suitable brand
Vortex mixer	Any suitable brand
Microcentrifuge	Any suitable brand
Functioned laminar flow hood	Any suitable brand

Note : Appropriate work space (sample preparation and post-PCR reaction should be separated.)

General Workflow of Designing a Nucleic Acid Assay

Identify assay application

- SNP analysis
- Rearrangement Detection
- Insertion/Deletion Detection
- Sequence Detection (Pathogen, etc.)
- DNA methylation

Design PCR primer/conditions for efficient amplification

- Primer composition for standard or mutant enrichment PCR or RT-PCR
- Determine PCR program for efficient and specific target amplification

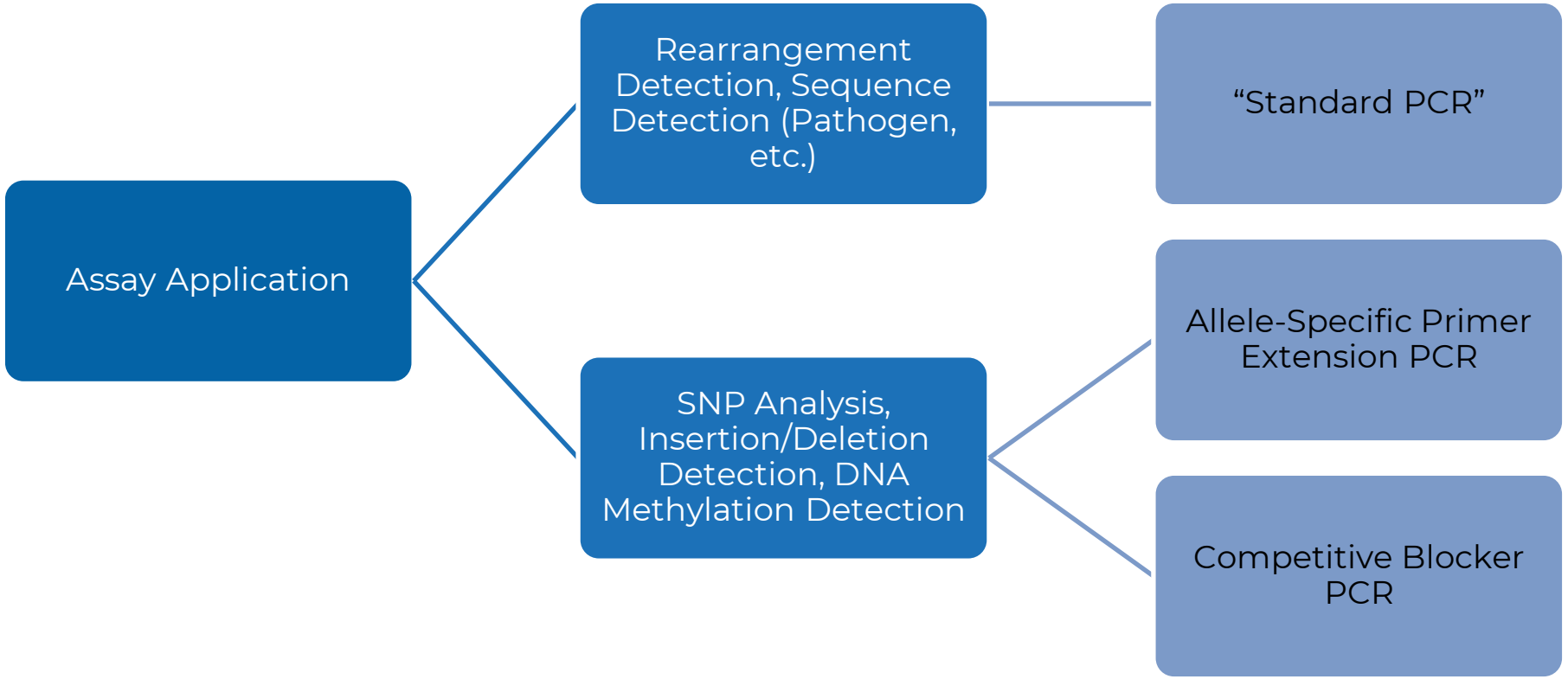
Design probes and hybridization condition

- Design composition of probes
- Determine hybridization conditions (temperature, ,duration, buffer, etc.)

Optimization & Validation

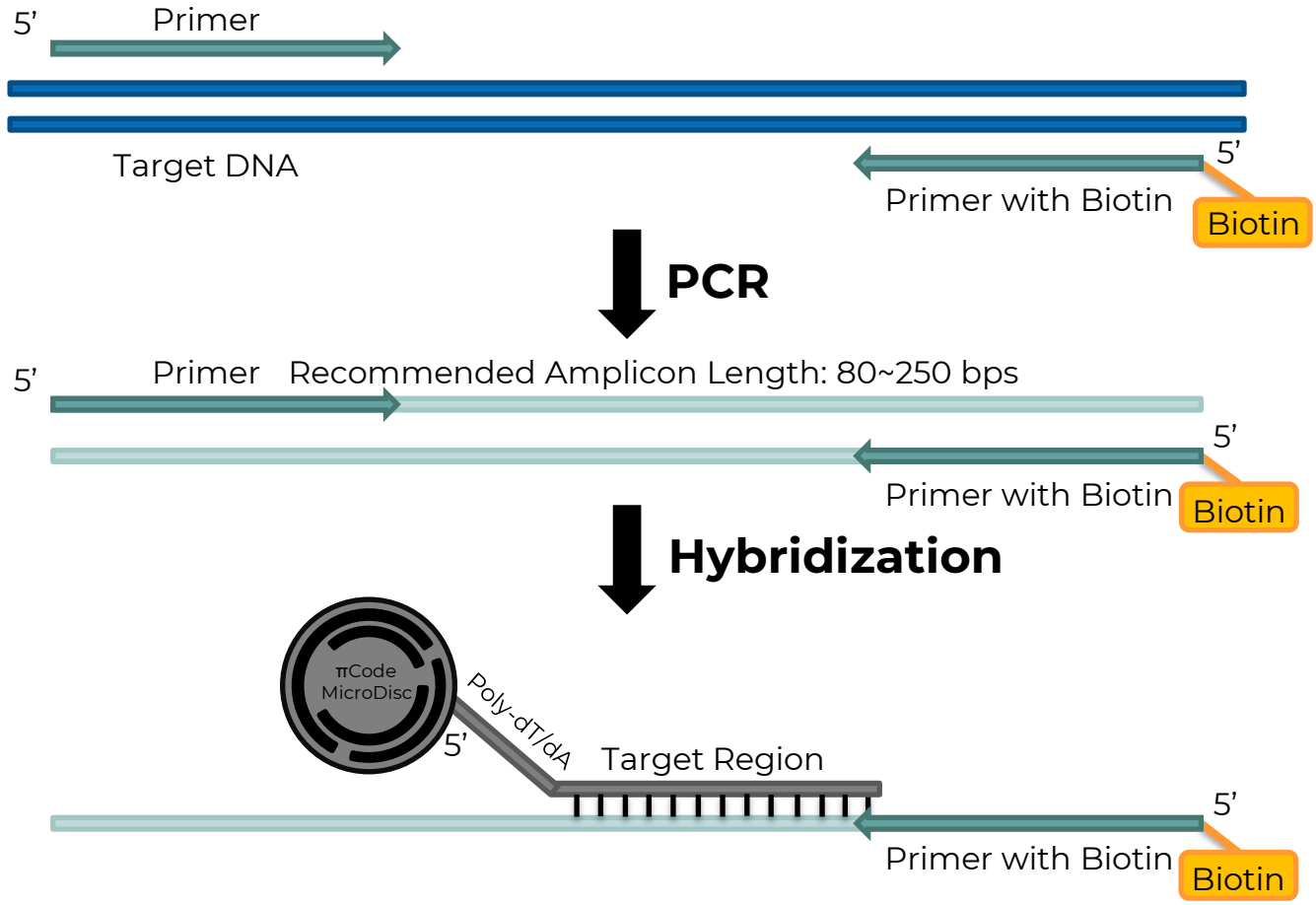
- Optimize detection by improving PCR and hybridization conditions
- Determine LoB, LoD, Accuracy, Precision & Reproducibility etc.
- Validate assay by comparison to "Gold Standard" methods

Determine Nucleic Acid Assay Format*

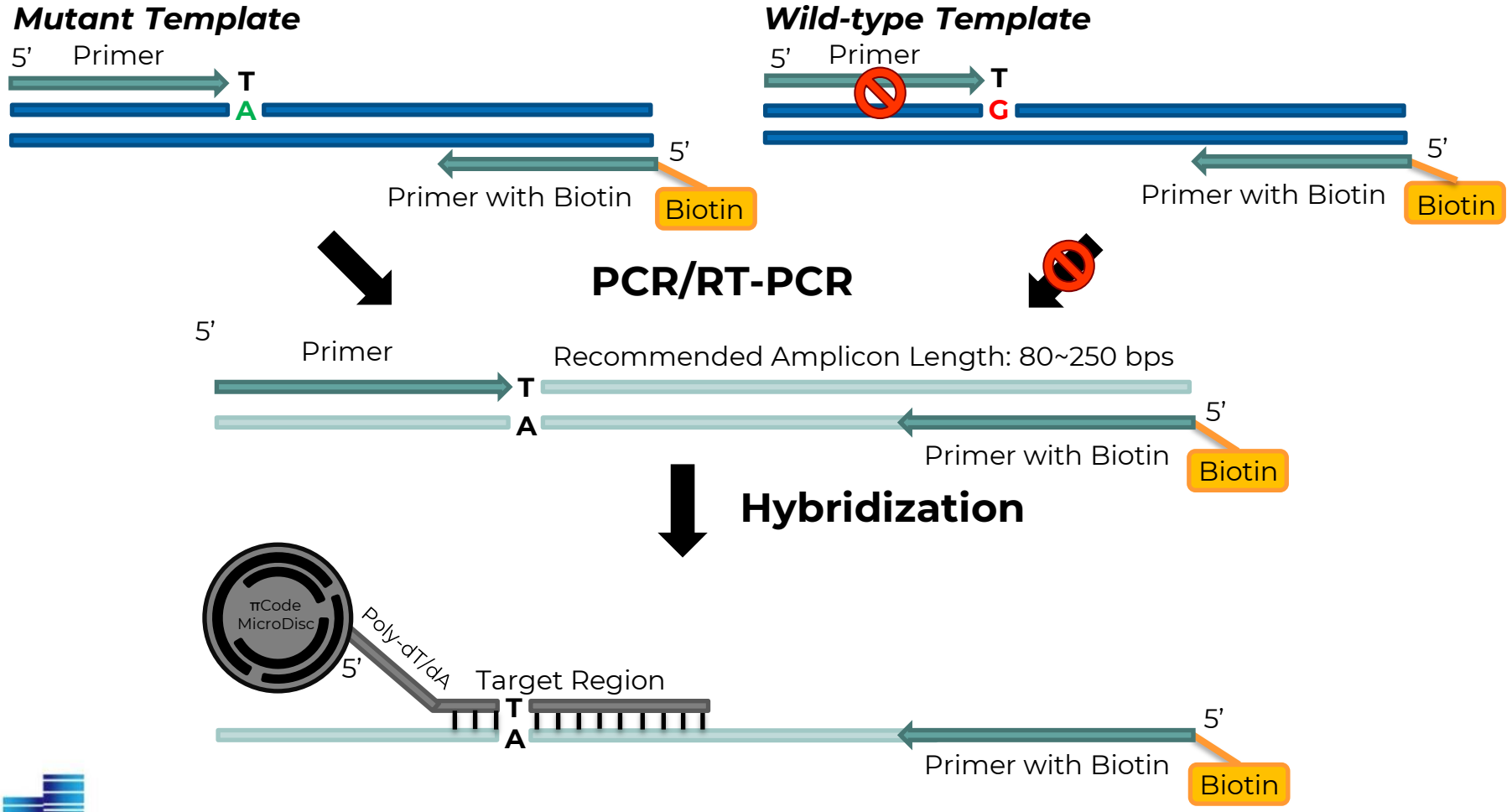


* PlexBio πCode MicroDisc technology supports alternative amplification or mutant enrichment methodologies

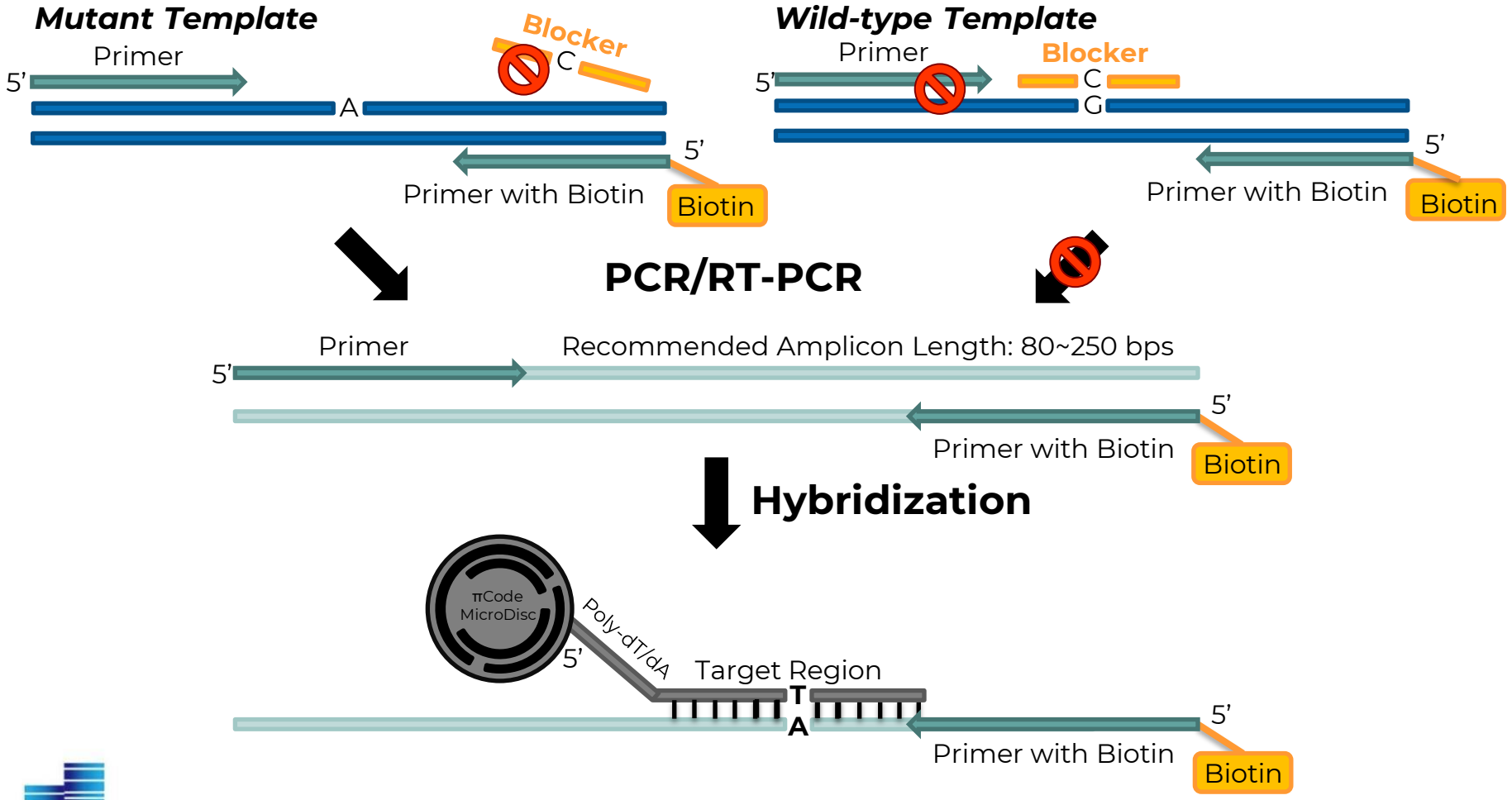
Standard PCR



Allele-specific Primer Extension PCR

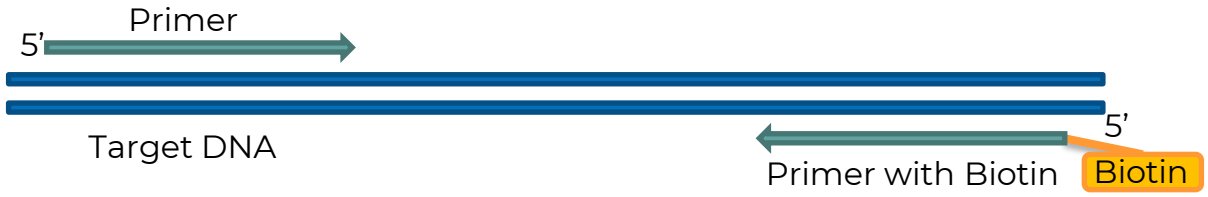


Competitive Blocker PCR

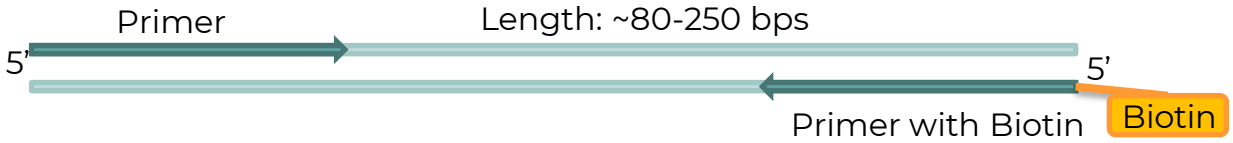


Primer Design

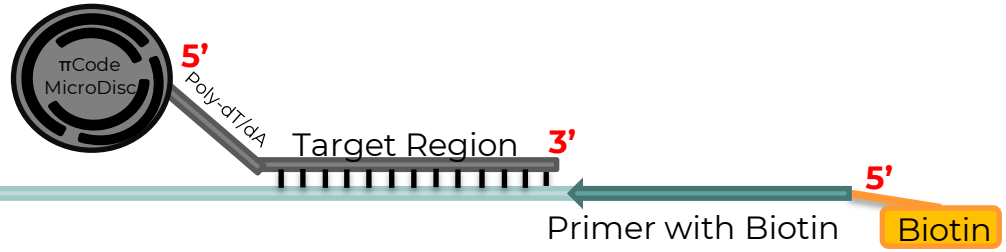
- Primers should be designed to amplify specific target regions.
- One of paired primers should be biotin-labeled at 5' end.



- Recommended Amplicon Length: ~80 to ~250 bps



- Biotin-labeled strand should be in **reverse** direction relative to the probe.

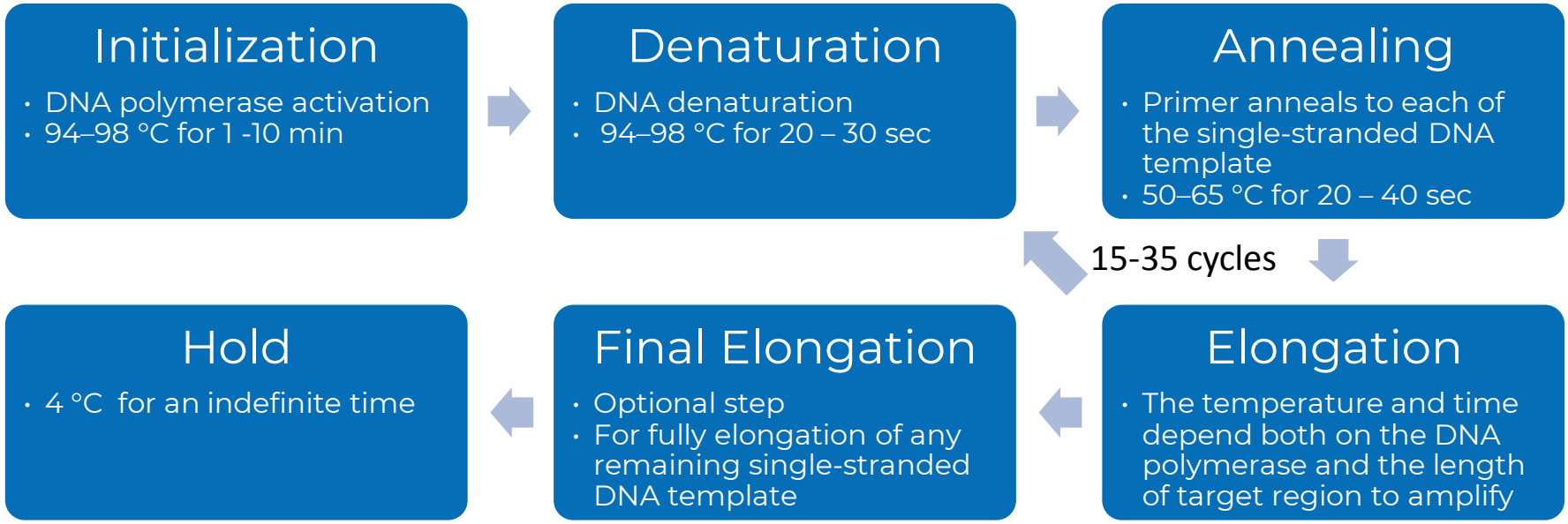


Primer Design

- Follow standard guidelines for primer design
  - All primer sets should obtain similar melting temperature. The temperature difference should not be more than 5°C.
  - Primers should not include complementary sequences to each other to avoid primer-dimer.
  - Primers should not have complementary reverse sequences to all the probes to avoid cross-reactivity.
  - Primers should not have secondary structures, like hairpin.
  - If multiple genomic regions are amplified, amplicons should be designed to be similar in size.
- Primers should be dissolved or diluted with TE buffer or ddH_2O, and stored as individual stock at -20°C or lower.

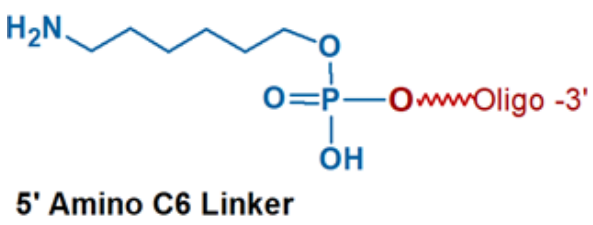
PCR Condition

- PCR conditions and primer compositions must be empirically optimized for each target. Synthetic or plasmid-derived templates with defined sequences as initial template are recommended.
- The individual steps of common PCR program are as follows:

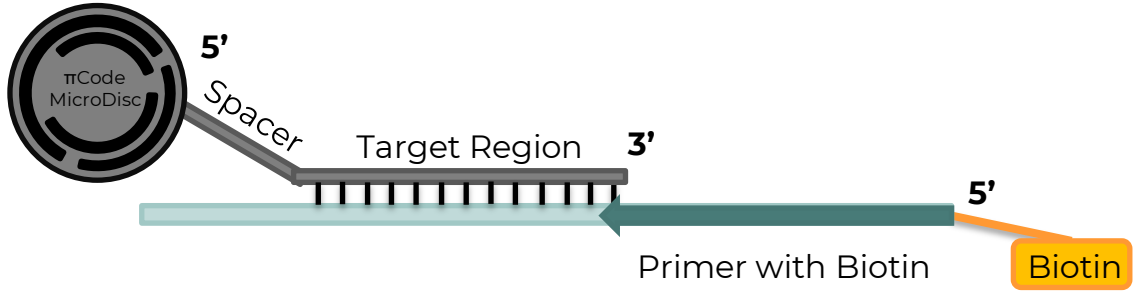


Probe Design

- C6 amino modified at 5’ end followed by a spacer element consisting of several unspecific nucleotides (i.e. poly-dA or poly-dT sequence); and the target sequence at 3’ end.



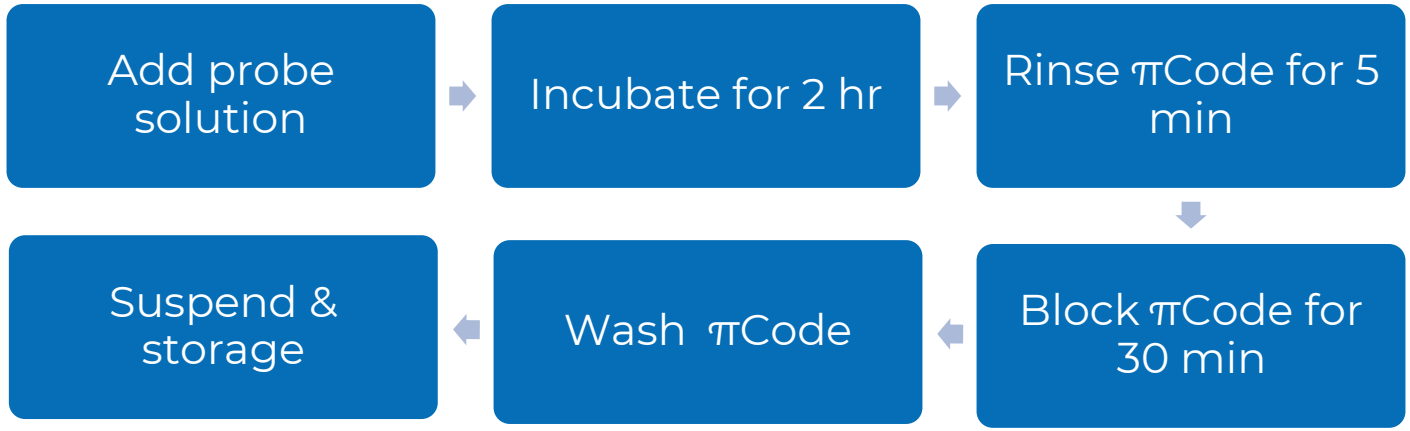
5' Amino C6 Linker



- A total length of probe (including unspecific poly-dA/dT sequence) is recommended to be **20~35 nt**
- Probes should be dissolved or diluted with **ddH_2O**, and stored as individual stock at -20˚C or lower.

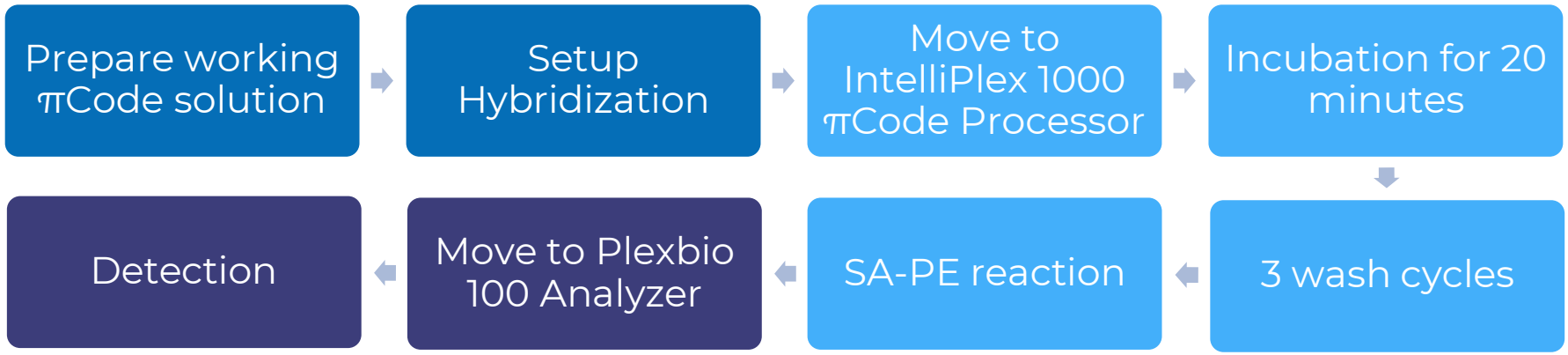
Coupling Workflow

- Total procedure time < 3.5 hours; hands-on time < 1 hours
- Suggested starting concentration of DNA probe solution is **3 μM in ddH2O**. The concentration should be optimized individually for each oligonucleotide.
- Up to 20 different coupling reactions can be processed in parallel for a proficient operator.
- See ***X-2_πCode Coupling Procedure*** for details.



Hybridization Conditions

- The performance of each DNA probe used must be validated individually before proceeding to multiplex assay. Please refer to user manual of πCode MicroDisc for further instruction.
- The suitable condition of the hybridization processes is largely dependent on the sequence and length of the DNA probes. Users have to optimize the conditions based on the experiment results.
- The following protocol is an example.



Hybridization Conditions

1. Prepare a working πCode solution by mixing selected coupled πCode MicroDisc stocks to a final concentration of **5 Disc/ID/µL** in Storage Buffer. 20 µL of the working πCode MicroDisc solution is recommended for each reaction.

Calculation example:

*If **5** different πCode MicroDiscs with stock concentration of **100** Discs/µL are selected and **10** reactions* are required:*

Total volume of working solution = *vol* per reaction × *num* of reaction

= 20 × 10 = **200** µL

Volume of each stock πCode = *Conc.* of working solution / *Conc.* of stock × total *vol*

= 5 / 100 × 200 = **10** µL

Volume of Storage Buffer = total *vol* - *vol* of each stock πCode × *num* of stock

= 200 - 10 × 5 = **150** µL

** The number of reaction is the number of wells to be tested. In addition, it is recommended to prepare excess amount of working solution.*

Hybridization Conditions

2. Aliquot **20** µL of the πCode MicroDisc solution prepared in Step 1 into wells of a **96-well Microplate** (*PlexBio, Cat No. 80025*). Vortex thoroughly before aliquoting.
3. Add **100** µL of **DNA Hybridization Buffer** *(PlexBio, Cat No. 80206)* into all tested wells.
4. Add **10** µL of samples (i.e. freshly denatured PCR amplicons) into selected wells.

NOTE: *The total volume added in each well should be less than 150 µL. Otherwise the liquid may overflow during incubation.*

Hybridization Conditions

5. Place the microplate into IntelliPlex 1000 πCode Processor and build a customized program for following hybridization process.

1. **Incubation**: 37˚C for 20 min
   - ■ Close lid while heating
   - ☐ Shake before reaching temp

NOTE: *The temperature and duration of incubation should be further optimized.*

2. **Washing**: 3 wash cycles
   - ■ 150 µL ☐ ELISA
   - ☐ Last Dispense Volume
   - ■ Magnetic
   - ■ Clean tip

NOTE: *The cycle of washing should be further optimized based on the assay design and results.*

3. **SA-PE Reaction**: 10 min

Hybridization Conditions

6. Add **SA-PE solution** (*PlexBio, Cat No. 80203*) into the V-tray.

   Calculation example:

   *the dead volume is **500** µL and the volume of SAPE solution per row is **400** µL.*

   For 3 rows reaction, the SA-PE solution needed is 400 µL x 3 rows + 500 µL = 1.7mL
7. Start the Hybridization program. Make sure there is sufficient amount of buffer in the bottles.
8. After all processes are completed, place the microplate into PlexBio 100 Analyzer for image recognition and fluorescent signal acquisition. Refer to user manual for detailed instruction.

Troubleshooting

Low fluorescent signal

1. Verify and optimize ***PCR amplification*** of the target sequence:
   - Optimize the design of the primers. Avoid secondary structure or primer dimer
   - Titrate the PCR primers input or increase the number of PCR cycles to optimize the PCR efficiency
   - Verify the size of PCR product on agarose gels and confirm absence of primer-dimer or unspecific fragments
   - Verify the sequence of PCR product by sequencing
   - Optimize PCR conditions (such as PCR cycle number, PCR temperatures & incubation times or primer concentration) to optimize the PCR efficiency

Troubleshooting

Low fluorescent signal

2. Verify and optimize ***hybridization conditions***:
   - Verify the design of probes. Avoid secondary structure or dimer
   - Verify coupling efficiency of probes to πCode MicroDisc by using serial titration of biotinylated oligonucleotide (complementary and reverse to the probe) for hybridization.
   - Change hybridization temperature, buffer and/or time
   - Verify the complementarity between probes and amplicon. Ensure that probe is in reverse direction to the amplicon strand containing biotin-labeled primer. Redesign the primers and/or probes if needed.
   - Ensure functional SA-PE solution is used. Make sure SA-PE solution is stored at 4˚C in the dark.

NOTE: *The fluorescent intensity of nonspecific background is around 2000 to 5000.*

Troubleshooting

High background signal

1. If high background is observed in one or a few πCode sets, verify the specificity of probes sequence and recouple. In some cases, re-synthesizing/ -purifying the probe can solve the unspecific background problems. Always ensure blocking step is performed during the coupling process of probes to πCode MicroDiscs.
2. If high background occurs in all πCode sets, optimizing hybridization stringency (hybridization buffer/ hybridization temperature) may reduce the background. Always make sure to use sufficient and freshly prepared hybridization and wash buffer and ensure that the manifolds of IntelliPlex 1000 πCode Processor is not clogged.

Troubleshooting

High background signal

3. If high background persists, replace all coupling buffers and probes, clean the work area, and recouple the probes using only nuclease-free, filter pipette tips for transferring reagents.
4. If high background originates from PCR contamination, replace the PCR reagent, clean the working area and retest. Please see ***X-3_Lab Requirement*** for details.

Troubleshooting

Low specificity

1. Verify the sequence specificity of primers and probes
2. Verify PCR condition and specificity of amplicons
3. Increase hybridization temperature or shorten the “leaky” probe
4. Redesign primers/probes to target a more distinctive region

Low πcode count

1. Make sure to vortex πCodes thoroughly before adding to each well.
2. Microbes grow in wash buffer, ddH_2O or fluid path tubes in IntelliPlex 1000 πCode Processor, which results in severe πCode loss. Please wash the system with 70% ethanol and use fresh prepared wash buffer and ddH2O for hybridization. Please see ***P-3_Long-term Shutdown*** for instruction.

Thank You

PlexBio
6F-1, No. 351, Yangguang St.
Neihu District, Taipei City 11491, Taiwan
+866-2-2627-5878 | PlexBio.com
service@plexbio.com