Comprehensive Testing for neurofibromatoses, RASopathies, and tuberous sclerosis at UAB
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## BACKGROUND

The RASopathies are a genetically heterogeneous group of disorders caused by mutations in the genes involved in the Ras-MAPK pathway. As a group, the RASopathies are one of the largest groups of malformation syndromes known, affecting ~1:1,000 and include Neurofibromatosis type 1, Legius syndrome, Noonan syndrome, cardio-facio-cutaneous (CFC) syndrome, Noonan Syndrome with Multiple Lentigines (NSML/-LEOPARD) and Costello syndrome. Mutations in NF1 and SPRED1 are typically loss-of-function mutations and include the full spectrum of nonsense, missense, splice, frameshift, insertion-deletion, and copy number changes. Mutations in the other RASopathy genes are typically missense mutations or/and in-frame deletion/insertion of an amino acid.
The Ras/MAPK pathway can have a profound deleterious effect on development as it plays a key role in differentiation, growth, senescence, and dysregulation. Clinical features of the RASopathies include short stature; cardiovascular defects; cutaneous and pigmented findings; characteristic facies; skeletal and neurocognitive delays as well as a predisposition to neoplasia, both benign and malignant. The disorders have variable expressivity (individuals with the same disorder may show differing features and severity of symptoms, even within the same family). Some of the genes/mutations are not fully penetrant, therefore an individual may carry a mutation but not show any or only few signs of the syndrome. Moreover, features can change/progress with age, which makes it difficult to make an accurate clinical diagnosis. The RASopathies are inherited in an autosomal dominant manner. A parent who carries a mutated gene has a 50% chance of passing it on to every child, regardless of gender. An individual can carry a mutation either a) because (s)he inherited the mutation from a parent (parent clinically affected or “non-penetrant”), or b) because the mutation arose “de novo” in the egg or sperm from which the individual developed. Sometimes, the mutation occurred “post-zygotically”, i.e. during development and in these individuals the mutation may not be present in every cell of the body, typically resulting in a more mild phenotype (mosaicism).

**Neurofibromatosis type 1**

The *NF1* gene, cloned in 1990, was the first gene within the Ras-MAPK pathway shown to be associated with an autosomal dominant disorder, *Neurofibromatosis type I* (NF1). NF1, affects ~1/3000 individuals worldwide, with half of the patients being sporadic. NF1 is notorious for its phenotypic variability and is a progressive disorder with more signs developing with time. Although the NIH criteria enables clinicians to make a diagnosis in the majority of classically affected cases, diagnostic criteria are not met until a given age is reached. Atypical presentations also exist with patients not yet fulfilling NIH criteria by adulthood. The mutational spectrum of *NF1* is very complex and includes a wealth of unusual splice mutations affecting exon sequences as well as deep intronic mutations resulting in exonization of intronic sequences at the mRNA level.

**Legius syndrome**

Germline loss-of-function mutations in *SPRED1*, a negative regulator of the RAS-MAPK pathway, cause a neurofibromatosis type 1-like phenotype, first described in 2007 (*Legius syndrome*). Patients present with multiple café-au-lait spots with or without skinfold freckling. Other typical NF1 associated features (Lisch nodules, bone abnormalities, neurofibromas, optic pathway gliomas) are systematically absent. However, in some patients Noonan-like features are present.

**Noonan syndrome**

*Noonan syndrome* (NS), *Noonan Syndrome with Multiple Lentigines* (NSML, aka LEOPARD) and *Noonan syndrome with “loose anagen hair”* are autosomal dominant disorders affecting ~1:1,000-2,000 individuals. Patients present with craniofacial features and a variable clinical phenotype including congenital heart defects, reduced growth, bleeding disorders (NS), and variable degrees of neurocognitive delay. Patients with NSML also have multiple lentigines, genital abnormalities and sensorineural deafness. sparse slow growing hair.
Patients with NS also have an increased cancer predisposition. Genes associated with NS and NSML are \textit{PTPN11}, \textit{PPP1CB}, \textit{KRAS}, \textit{SOS1}, \textit{RAF1}, \textit{NRAS}, \textit{BRAF}, \textit{MAP2K1}, \textit{CBL}, \textit{RIT1}, \textit{RASA2} and \textit{SOS2}. The \textit{SHOC2} and \textit{PPP1CB} gene is associated with NS with “loose anagen hair” or sparse slow growing hair (Gripp K. et all, 2016).

\textbf{Cardio-Facio-Cutaneous syndrome}

\textbf{Cardio-Facio-Cutaneous syndrome} (CFC) is a rare condition with genetic and phenotypic overlap with NS. Clinical features include craniofacial features similar to those found in NS, neurocognitive delay, failure to thrive, congenital heart defects, epilepsy and a wide range of ectodermal manifestations. Four genes have been associated with CFC: \textit{BRAF, MAP2K1, MAP2K2} and \textit{KRAS}.

\textbf{Costello syndrome}

\textbf{Costello syndrome} (CS), caused by activating \textit{HRAS} mutations, is a very rare condition with the following key features: coarse facial features, severe feeding difficulty, mild to moderate intellectual disability, relative macrocephaly and short stature, high incidence of cardiac abnormalities and malignancy. Differentiation of CS from other RASopathies, particularly CFC may be difficult especially early in life.

\textbf{Peripheral Nerve Sheath Tumors}

Peripheral nerves connect the brain and spinal cord to the other parts of the body. Peripheral nerve sheath tumors develop from the Schwann cells, i.e. cells covering the peripheral nerves, and can be divided into benign and malignant tumors, although ~90% of such tumors are benign.

Nerve sheath tumors include neurofibromas and schwannomas. They can occur as solitary lesions; however, if multiple develop, this may indicate the presence of a hereditary predisposition. Neurofibromas are common, benign tumors, composed of a complex heterogeneous mixture of cells, and the presence of 2 or more such tumors (cutaneous, subcutaneous or plexiform) is a diagnostic sign of neurofibromatosis type 1, with two \textit{NF1} hits found specifically in the Schwann cells only. Whereas cutaneous neurofibromas never undergo malignant transformation, subcutaneous and plexiform neurofibromas may undergo malignant transformation (Evans G. et al, 2002).

Schwannomas are more homogeneously composed benign tumors and if multiple such tumors are present, they may indicate the presence of neurofibromatosis type 2 (NF2) or schwannomatosis. The location of the schwannomas differs between \textit{NF2} and \textit{SMARCB1} or \textit{LZTR1}-related schwannomatosis with (bilateral) vestibular schwannomas typically present in classic NF2, and intradermal and non-intradermal schwannomas also frequently found. Schwannomatosis-associated schwannomas are usually non-intradermal and non-vestibular, however overlap clearly exists and genetic characterization can be an important tool to distinguish between both disorders.

Some types of schwannomas mimic neurofibromas and vice versa. They are called myxoid schwannomas and hybrid neurofibromas/schwannomas.
In addition, one patient with a KRAS germline mutation p.K5E and multiple diffuse schwannomas was described and a KRAS mutation p.G12S was found in 1/40 sporadic schwannomas. Furthermore, several patients with Noonan syndrome with multiple lentigines (NSML), also fulfilling criteria for NF1, carrying a mutation in the PTPN11 gene but not in the NF1 gene, have recently been described. The patients had plexiform neurofibromas, large dumbbell spinal tumors and hypertrophic peripheral nerves.


**Capillary/Arteriovenous Malformation syndrome/Parkes Weber syndrome**

Capillary malformation-arteriovenous malformation syndrome (CM-AVM) is a disorder of the vascular system, which is the body’s complex network of blood vessels. Parkes Weber syndrome is characterized by congenital vascular abnormalities known as capillary malformations and arteriovenous fistulas (AVFs). Some vascular abnormalities seen in Parkes Weber syndrome are similar to those that occur in capillary malformation-arteriovenous malformation syndrome (CM-AVM). CM-AVM and some cases of Parkes Weber syndrome are caused by mutations in the RASA1 gene. (Am J Hum Genet. 2003;2003;73:1240–9).

**Neurofibromatosis Type 2**

Neurofibromatosis 2 (NF2) is an autosomal dominant disorder which is characterized by bilateral vestibular schwannomas with associated symptoms of tinnitus, hearing loss, and balance dysfunction. Almost all affected individuals develop bilateral vestibular schwannomas by age 30 years. Affected individuals may also develop schwannomas of other cranial and peripheral nerves, meningiomas, and ependymomas. NF2 is the only gene in which pathogenic variants are known to cause neurofibromatosis 2, however there are some overlapping features in NF2 and Schwannomatosis.

**Schwannomatosis/ Multiple Schwannomas**

Schwannomas are benign nerve sheath tumors. These tumors are nearly always benign but can cause significant pain. Isolated schwannomas are common in the population, but the development of multiple non-intradermal schwannomas (in the absence of bilateral vestibular schwannomas, congenital cataracts or ependymomas, typically associated with constitutional NF2) is rare. Presence of multiple non-intradermal schwannomas, in the absence of a family history of NF2, can be found in individuals with mosaic Neurofibromatosis type 2, or in individuals carrying germline mutations in either SMARCB1 or LZTR1.
People with schwannomatosis do not typically develop vestibular schwannomas, ependymomas, meningiomas (typically associated with NF2), nor neurofibromas or astrocytomas (associated with NF1). However, clinical overlap with neurofibromatosis type 2 exists: some SMARCB1-positive patients have developed meningiomas or a unilateral vestibular schwannoma; and some LZTR1-positive patients have been reported with a unilateral/bilateral vestibular schwannoma. Whereas both NF2 and schwannomatosis present with variable expressivity, penetrance for NF2 is close to 100%, whereas non-penetrance is well documented in schwannomatosis, although the exact frequency is not known.

**Meningiomatosis/Multiple Meningiomas**


Besides NF2, germline SMARCB1 mutations have been identified in patients with meningiomas (with or without schwannomas) (van den Munckhof P et al, 2012: Neurogenetics 13:1-7). Furthermore, germline mutations in SUFU have been found in some families with meningiomatosis (Aavikko M et al, 2012: Am. J. Hum. Genet. 91:520-6).

Furthermore, SMARCE1 germline mutations are associated with a tumor-predisposition syndrome with patients having an increased risk for spinal and intracranial clear cell meningiomas (Smith M et al, 2014: J. Path. 234:436-40). Clear cell meningiomas occur more frequently in young people and are defined as WHO grade 2, due to their more aggressive behavior.

As multiple meningiomas can be a presenting sign of different tumor-predisposition syndromes with overlapping features, a clinical diagnosis might be challenging in some individuals, making a panel-based test more cost-effective.

**Rhabdoid Tumor Predisposition syndrome**

Rhabdoid tumors are rare, aggressive childhood cancers that most often develop in the kidney (Malignant Rhabdoid Tumor, MRT) and central nervous system (Atypical Teratoid/Rhabdoid Tumor, AT/RT). These lesions can occur spontaneously or as part of hereditary Rhabdoid Tumor Predisposition Syndrome (RTPS). In comparison to sporadic isolated rhabdoid tumors, the syndromic form is associated with an increased risk of developing multiple tumors at younger ages and schwannomas (benign nerve sheath tumors) that present primarily in adulthood. (Sevenet N. et al, 1999: Hum. Mol. Genet. 8:2359-68).

**Tuberous Sclerosis Complex**

Tuberous sclerosis complex (TSC) is a rare autosomal dominant disorder involving abnormalities of the skin, brain, kidney, heart and lungs. CNS tumors are seen commonly. Heterozygous pathogenic variants can be identified in 75%-90% of individuals who meet the clinical diagnostic criteria for TSC (Northrup H. et al, 2013: Ped. Neurology 49:243-4). Among those in whom a pathogenic variant can be identified, pathogenic variants in TSC1 and TSC2 are found in 31% and 69% of cases, respectively (Ozgur et al. Eur J Hum Genet. 2005;13:731–41)
Some individuals with a clinical diagnosis of one of the neurofibromatoses and/or RASopathies have been found to carry a mutation in a gene that was not considered to be consistent with their clinical diagnosis. Examples include \( \text{BRAF} \) variants reported in individuals with a clinical diagnosis of Noonan syndrome, a \( \text{SOS1} \) variant in an individual with CFC (Nystrom AM et al, 2008), \( \text{PTPN11} \) mutations in individuals with paraspinal neurofibromas (Conboy E. et al, 2015), and an \( \text{NF1} \) missense mutation in patients with Noonan-like features and no neurofibromas (Rojnueangnit K et al, 2015). In addition, some genes are associated with more than one syndrome (\( \text{PTPN11}, \text{KRAS}, \text{BRAF}, \text{RAF1}, \text{NF1} \)). Therefore, the comprehensive approach of simultaneously testing all 17 genes available in the Expanded NF1-RASopathy NGS panel (please see pg 8 for more detail) in some individuals eliminates the need to determine which genes to test based on an individual’s clinical signs.

Given the spectrum of genes involved in the RASopathies, NF2, Schwannomatosis, Tuberous sclerosis complex and the resulting phenotypic variability and/or overlap, the initial diagnosis is often clinical. Molecular genetic testing is used for confirmatory diagnosis as well as to help with diagnosis in ambiguous cases. Molecular diagnoses can play a significant role in helping to overcome the limitations of the clinical diagnoses with progressive accumulation of genotype-phenotype correlation data. Moreover, it can also aid in discovery of potential therapeutic targets and improve patient management.

For further information, the review articles/references on page 14 can be consulted.

**TESTING STRATEGY - many options at UAB**

**DNA-based Testing**

**TEST OPTIONS**
- NF1-only by NGS
- NF1/SPRED1-only by NGS
- Expanded NF1-RASopathy NGS panel (17 genes)
- Non-NF1 RASopathy NGS panel (16 genes)
- NF2-only by NGS
- Schwannomatosis/Multiple Schwannomas panel by NGS (3 genes)*
- Meningiomas/Multiple meningeomas panel by NGS (4 genes)*
- Peripheral Nerve Sheath Tumor panel (6 genes)*
- Capillary Malformation/Parkes Weber (RASA1)
- Rhabdoid Tumor Predisposition syndrome (SMARCB1)*
- Tuberous Sclerosis Complex (2 genes)*

**SPECIMEN TYPES**
- Blood (2-3ml EDTA; no time limitations associated with receipt)
- Saliva (OGR-575 DNA Genotek; kits are provided upon request)
- DNA (extracted from lymphocyte cells, a minimum of 3µg, O.D. value at 260:280nm ≥1.8)
- Fresh/Frozen (>60% pure tumor cells, 3-5mm-cubed in size) tumor available for any test notated with an astrisk (*)

**TURNAROUND TIME**
- 25 working days
**NF1-only by NGS**

**Candidates for this test:** patients with classic NF1 *including* the presence of cutaneous neurofibromas or Lisch nodules, as no genetic heterogeneity demonstrated so far associated with this phenotype.

The **NF1-only by NGS** involves sequencing as well as **deletion/duplication analysis** of the entire coding NF1 region plus the alternatively spliced exons 9br, 23a and 48a (60 exons total). The test uses an extensively customized and optimized set of Agilent HaloPlex capture probes, followed by sequencing of overlapping amplicons within the regions of interest using Illumina sequencing chemistry. Each coding exon plus ~50bp of flanking intronic sequence are simultaneously sequenced. 5' and 3' untranslated sequences are not included. **The average coverage is >2000x with >99.8% of the NF1 coding region ≥350x and 100% ≥200x, allowing detection of very low level mosaicism, down to 3-5% MAF respectively (regions covered by ≥350x respectively ≥200x).** Variant and copy number calls are made using a unique bioinformatics pipeline detecting all types of mutations including single nucleotide substitutions, indels, and frameshifts caused by deletion/duplication up to 112bp.

Based on >15 years of experience with comprehensive RNA-based NF1 testing, we designed the **customized and optimized NGS NF1-component** of the assay to comprise **all regions** encountered through analysis of >15,000 unrelated individuals including >8,100 NF1-mutation-positive individuals carrying 1 out of >3,100 different *unique NF1* mutations identified in the UAB MGL cohort. Included in the NGS assay are the regions covering >65 different deep intronic splice mutations (which reside beyond the +/-50 intronic base pairs that flank all exons). Validation of the full panel included, besides substitutions (missense, nonsense, splice variants), the most challenging mutations such as insertions/deletions/duplications of 1-112bp (~25% of the UAB NF1 cohort) and one-to-multiple exon deletions/duplications (~2.8% of the UAB NF1 cohort). The analytical sensitivity of our NGS testing approach was 100% for substitutions as well as insertion/deletions up to 112bp. This panel has not yet been validated to identify deletions/duplications >112bp and <1 exon, but such mutations have not yet been found in the UAB cohort, and therefore are likely very rare. The panel has been validated for the detection of *germline* (heterozygous) single-exon deletions/duplications as well as multi-exon deletions/duplications, however *mosaic* single-exon deletion/duplications validation is still pending. *Single* exon deletions/duplications are present in ~0.45% of NF1-positive patients from the UAB cohort with 9% of these individuals being mosaic (~0.045% of all in the UAB NF1-positive cohort). Detection of *Alu/LINE* insertions, identified in 0.25% of patients from the UAB NF1-positive cohort, has not yet been validated using the current NGS approach.

With the **largest dataset of NF1 genotypes matched with phenotypes**, any genotype-phenotype correlations identified will be reported in real time.

**Confirmatory testing of reportable variants** is performed by Sanger sequencing or other orthogonal methods.

For **novel NF1 variants of unknown significance**, we offer free of charge targeted RNA-based testing to assess the effect of the variant on splicing and enhance the correct classification/interpretation.
Relevant family members of a proband with any (novel or previously identified) variant of unknown significance are offered free of charge targeted analysis as long as accurate phenotypic data are provided by a health care professional to enhance the interpretation. There is no limitation to the number of relatives that can be tested free of charge.

Mosaicism is often present in sporadic patients with an NF1 microdeletion and has important repercussions for counseling. Free of charge evaluation by FISH analysis on 200 interphase chromosomes is offered in such cases.

**NF1/SPRED1-only by NGS**

**Candidates for this test:** patients with multiple CALMs w/wo skinfold freckling and no other typical NF1 associated features (Lisch nodules, bone abnormalities, neurofibromas, optic pathway gliomas).

The DNA-based NF1/SPRED1-only by NGS involves sequencing as well as deletion/duplication analysis of the entire coding NF1/SPRED1 regions plus the alternatively spliced NF1 exons 9br, 23a and 48a (67 exons total). The test uses the same approach as detailed above (see: NF1-only by NGS). The average coverage of the NF1/SPRED1-only by NGS panel is >2000x with >99.8% of the NF1 coding region ≥350x and 100% ≥200x, allowing detection of very low level mosaicism, down to 3-5% MAF respectively (regions covered by ≥350x respectively ≥200x).

**Expanded NF1-RASopathy by NGS**

**Candidates for this test:** patients with clinical features suggestive of either NS, NSML, CFC, NF1, Legius syndrome or Noonan-like syndrome; patients with a clinical diagnosis of any of these syndromes that previously tested negative in a subset of the genes included in this panel; patients with a diagnosis of Costello syndrome but no HRAS mutation previously identified.

The Expanded NF1-Rasopathy panel by NGS involves the simultaneous sequencing of 17 genes: NF1, SPRED1, PTPN11, PPP1CB, BRAF, CBL, HRAS, KRAS, NRAS, MAP2K1, MAP2K2, RAF1, RIT1, RASA2, SHOC2, SOS1 and SOS2 (282 exons). The test uses the same approach as detailed above (see: NF1-only by NGS). The average coverage is >2000x with >99.8% of the NF1 coding region ≥350x and 100% ≥200x, allowing detection of very low level mosaicism, down to 3-5% MAF respectively (regions covered by ≥350x respectively ≥200x) for the NF1 gene. For the remainder of the genes, the average coverage is 2000x with >99.5% of the coding region covered at ≥350x and 99.2% covered at 200x. The minimum coverage for any additional areas is >30x. Variant and copy number calls are made using a unique bioinformatics pipeline detecting all types of mutations including single nucleotide substitutions, indels and frameshifts caused by deletion or duplication up to 112bp. Deletion/duplication analysis for NF1/SPRED1 is included in this test, as such mutations are a part of the mutation spectrum for these conditions. Deletion/duplication analysis for the other 14 genes on this panel is not offered as current empirical and biological evidence is not sufficient to allow the conclusion that an altered copy number of these genes is a mechanism critical for the phenotype associated with the Rasopathies.
**Non-NF1 RASopathy NGS panel**

Candidates for this test: patients with clinical features suggestive of either NS, NSML, CFC, Legius syndrome or Noonan-like syndrome with no mutation previously found by comprehensive RNA-based NF1+/- SPRED1 testing.

The **non-NF1 Rasopathy by NGS** involves the simultaneous sequencing of 16 genes: SPRED1, PTPN11, PPP1CB, BRAF, CBL, HRAS, KRAS, NRAS, MAP2K1, MAP2K2, RAF1, RIT1, RASA2, SHOC2, SOS1 and SOS2 (220 exons). The test uses the same approach as detailed above (see: NF1-only by NGS). The **average coverage is 2000x with >99.5% of the coding region covered at ≥350x and 99.2% covered at 200x.** The minimum coverage for any additional areas is >30x. Variant and copy number calls are made using a unique bioinformatics pipeline detecting all types of mutations including single nucleotide substitutions, indels and frameshifts caused by deletion or duplication up to 112bp. **Deletion/duplication** analysis for *SPRED1* is included in this test, as such mutations are a part of the mutation spectrum for this gene. Deletion/duplication analysis for the other 15 genes on this panel is not offered as current empirical and biological evidence is not sufficient to allow the conclusion that an altered copy number of these genes is a mechanism critical for the phenotype associated

**NF2-only by NGS**

Candidates for this test: patients with bilateral vestibular schwannomas w/wo other typical NF2 associated features (congenital cataracts, ependymoma, facial weakness, etc).

The DNA-based **NF2-only by NGS** involves sequencing as well as deletion/duplication analysis of the entire coding *NF2* regions. The test uses the same approach as detailed above (see: NF1-only by NGS). The **average coverage is 2000x with >99.5% of the coding region covered at ≥350x and 99.2% covered at 200x.** The minimum coverage for any additional areas is >30x. Variant and copy number calls are made using a unique bioinformatics pipeline detecting all types of mutations including single nucleotide substitutions, indels and frameshifts caused by deletion or duplication up to 112bp. Deletion/duplication analysis for *NF2* included in this test, as such mutations are a part of the mutation spectrum for these conditions.

**Rhabdoid Tumor Predisposition syndrome by NGS**

Candidates for this test: patients with rhabdoid tumors w/wo confirmed SMARCB1-loss identified by immunohistochemistry staining.

The DNA-based **Rhabdoid Tumor Predisposition syndrome by NGS** involves sequencing as well as deletion/duplication analysis of the entire coding *SMARCB1* regions. The test uses the same approach as detailed above (see: NF1-only by NGS). The **average coverage is 2000x with >99.5% of the coding region covered at ≥350x and 99.2% covered at 200x.** The minimum coverage for any additional areas is >30x.
Variant and copy number calls are made using a unique bioinformatics pipeline detecting all types of mutations including single nucleotide substitutions, indels and frameshifts caused by deletion or duplication up to 112bp. Deletion/duplication analysis for NF2 is included in this test, as such mutations are apart of the mutation spectrum for these conditions.

**Schwannomatosis/Multiple schwannomas Panel by NGS**
*Candidates for this test:* patients with multiple schwannomas w/wo vestibular schwannomas. This testing approach is developed to diagnose mosaic NF2, classic NF2, Schwannomatosis, and mosaic schwannomatosis.

The **Schwannomatosis/ multiple schwannomas NGS panel** involves the simultaneous sequencing of 3 genes: NF2, SMARCB1, and LZTR1. The test uses the same approach as detailed above (see: NF1-only by NGS). The **average coverage is 2000x with >99.5% of the coding region covered at ≥350x and 99.2% covered at 200x**. The minimum coverage for any additional areas is >30x. This allows for detection of very low level mosiacism by sequencing (as low as 8% of the alleles in all regions analyzed by NGS; >99% of the coding region does provide deeper coverage with the ability to identify substitution variants as low as 3% of the alleles). Variant and copy number calls are made using a unique bioinformatics pipeline detecting all types of mutations including single nucleotide substitutions, indels and frameshifts caused by deletion or duplication up to 112bp. Deletion/duplication analysis for NF2, SMARCB1, and LZTR1 is included in this test, as such mutations are a part of the mutation spectrum for these conditions.

**Mengiomatosis/Multiple meningiomas panel by NGS**
*Candidates for this test:* patients with clinical features suggestive of menigiomatosis or NF2 with the inclusion of mengiomias within their phenotype.

The **Meningiomatosis/Multiple meningiomas panel by NGS** involves the simultaneous sequencing of 4 genes: NF2, SMARCB1, SMARCE1, and SUFU. The test uses the same approach as detailed above (see: NF1-only by NGS). The **average coverage is 2000x with >99.5% of the coding region covered at ≥350x and 99.2% covered at 200x**. The minimum coverage for any additional areas is >30x. This allows for detection of very low level mosiacism by sequencing (as low as 8% of the alleles in all regions analyzed by NGS; >99% of the coding region does provide deeper coverage with the ability to identify substitution variants as low as 3% of the alleles). Variant and copy number calls are made using a unique bioinformatics pipeline detecting all types of mutations including single nucleotide substitutions, indels and frameshifts caused by deletion or duplication up to 112bp. Deletion/duplication analysis for NF2 and SMARCB1 is included in this test, as such mutations are a part of the mutation spectrum for these conditions. Deletion/duplication analysis for the other genes on this
panel is not offered as current empirical and biological evidence is not sufficient to allow the conclusion that an altered copy number of these genes is a mechanism critical for the phenotype associated with these conditions.

**Peripheral Nerve Sheath Tumor Panel by NGS**

**Candidates for this test:** patients presenting with both neurofibromas and schwannomas or peripheral nerve sheath tumors with mixed or unconfirmed cellularity w/ minimal additional findings meeting diagnostic criteria for any specific condition.

The **Peripheral Nerve Sheath Tumor Panel by NGS** involves the simultaneous sequencing of 6 genes: NF1, NF2, KRAS, LZTR1, PTPN11, and SMARCB1. The test uses the same approach as detailed above (see: NF1-only by NGS). The \textbf{average coverage is 2000x with 99.5\% of the coding region covered at 350x and 99.2\% covered at 200x.} The minimum coverage for any additional areas is >30x. This allows for detection of very low level mosiacism by sequencing (as low as 8\% of the alleles in all regions analyzed by NGS; >99\% of the coding region does provide deeper coverage with the ability to identify substitution variants as low as 3\% of the alleles). Variant and copy number calls are made using a unique bioinformatics pipeline detecting all types of mutations including single nucleotide substitutions, indels and frameshifts caused by deletion or duplication up to 112bp. Deletion/duplication analysis for NF1, NF2, SMARCB1, and LZTR1 is included in this test, as such mutations are a part of the mutation spectrum for these conditions. Deletion/duplication analysis for PTPN11 and KRAS not offered as current empirical and biological evidence is not sufficient to allow the conclusion that an altered copy number of PTPN11 and KRAS is a mechanism critical for the phenotype associated with these conditions.

**Tuberous Sclerosis Complex Panel by NGS**

**Candidates for this test:** patients with clinical features suggestive of Tuberous Sclerosis Complex.

The **Tuberous Sclerosis Complex panel by NGS** involves the simultaneous sequencing of 2 genes: TSC1 and TSC2. The test uses the same approach as detailed above (see: NF1-only by NGS). The \textbf{average coverage is 2000x with 99.5\% of the coding region covered at 350x and 99.2\% covered at 200x.} The minimum coverage for any additional areas is >30x. This allows for detection of very low level mosiacism by sequencing (as low as 8\% of the alleles in all regions analyzed by NGS; >99\% of the coding region does provide deeper coverage with the ability to identify substitution variants as low as 3\% of the alleles). Variant and copy number calls are made using a unique bioinformatics pipeline detecting all types of mutations including single nucleotide substitutions, indels and frameshifts caused by deletion or duplication up to 112bp. Deletion/duplication analysis for TSC1 and TSC2 is included in this test, as such mutations are a part of the mutation spectrum for these conditions.
RNA-based Testing

TEST OPTIONS
• RUSH RNA-based NF1 and DNA-based SPRED1 testing on blood
• RNA-based NF1 testing on blood
• RNA-based NF1/SPRED1 testing on affected tissues

SPECIMEN TYPES
• Fresh EDTA blood sample, to arrive in the lab <60-70 hours after blood draw
• Biopsies of café-au-lait macules (CALM) and/or neurofibromas

RUSH RNA-based NF1 and DNA-based SPRED1 testing
Candidates for this test: patients who need the most sensitive and specific test with the fastest turnaround time.

The RUSH RNA-based NF1 and DNA-based SPRED1 testing on blood requires a fresh EDTA blood sample, to arrive in the lab <60-70 hours after blood draw. DNA is extracted and in addition, a short term phytohemagglutinin-stimulated lymphocyte culture is initiated and used as starting material to extract RNA. The complete NF1 coding region is analyzed by a cascade of complementary mutation detection techniques, including RT-PCR, direct sequencing of cDNA fragments, microsatellite marker analysis, copy number analysis by MLPA and interphase FISH (if needed), enabling identification of the mutation in ~95% of non-founder patients fulfilling the NIH diagnostic criteria (Messiaen et al, 2000; Messiaen and Wimmer, 2008).

RNA-based NF1 testing allows finding deep intronic splice mutations through their observed effect on splicing. These splice mutations would not be detected if a “simple” exon-by-exon DNA-based (NGS/Sanger) sequencing approach is used. During the >15 years we have offered comprehensive RNA-based NF1 testing on blood, we have identified >65 different deep intronic splice mutations: together they account for 2.5% of all pathogenic mutations identified in the NF1 UAB cohort. Please note, however, that all known deep intronic splice mutations have been incorporated in the customized UAB NGS assay described above.

In addition, all coding exons and flanking intronic sequences of the SPRED1 gene are analysed by bidirectional sequencing and deletion/duplication analysis is performed using MLPA. Turnaround time for this test is 15 working days.
**RNA-based NF1 testing**

**Candidates for this test:** non-founder patients with a) clearcut clinically documented classic NF1; b) from a clinically documented multi-generation (minimum 3 generations) family; c) who tested negative by the MGL NF1-only by NGS assay and d) in whom a translocation has been excluded by cytogenetic analysis. The latter patients will receive free of charge reflex RNA-based NF1 testing, which should allow to detect a possible deep intronic splice mutation not previously identified in the UAB cohort and not reported elsewhere, or a possible Alu/LINE insertion or other exotic complex mutation. Based on current data, such mutations account conservatively for <0.25% of familial cases.

NF1 testing is performed as described in the previous section.

**RNA-based NF1/SPRED1 testing on cultured cells from affected tissues**

**Candidates for this test:** Individuals suspected to have segmental NF1, with symptoms restricted to a defined area of the body; sporadic patients who have (mild) non-localized symptoms of NF1 but in whom no NF1 mutation was identified in the blood lymphocytes and may have disease due to a postzygotic mutation; reflex testing for familial or sporadic patients with a first hit mutation refractory to detection by RNA- or DNA/NGS assay.

The RNA-based NF1/SPRED1 testing on cultured cells from affected tissues is offered starting from biopsies of café-au-lait macules (CALM) and/or neurofibromas. Melanocytes cultured from CALMs and Schwann cells cultured from neurofibromas are the starting material for the cascade of complementary mutation detection techniques described in the previous section on “Comprehensive RNA-based NF1 testing on blood” (Maertens et al, 2007, De Schepper et al, 2007). In addition, for patients with only pigmented features (CALMs w/wo skinfold freckling but no neurofibromas), and no NF1 mutations found in the melanocytes (no first or second hit mutations), the SPRED1 gene is analysed at no additional charge (sequencing and deletion/duplication analysis), as these patients may have mosaic Legius syndrome. As a result of this test, if features are NF1 or SPRED1-related, a common first hit is identified in both biopsies and a different second hit is identified in every anatomically different biopsy evaluated. If no mutations are identified despite full analysis on 2 biopsies with successful cultures, (mosaic) NF1/Legius syndrome is very unlikely (<0.1%).

We require a minimum of 2 biopsies (2-3 mm punch biopsies) from anatomically different locations. Please contact us at medgenomics@uabmc.edu or 205-934-5562 to set up a time to discuss your patient prior to taking biopsy/biopsies in your patient, so we can provide individualized advice and ship out appropriate collection/transport media and forms prior to the procedure.

**Please contact us if you have questions or want to discuss genetic testing needs for your patients. We will be happy to help.**
References


MEDICAL GENOMICS LABORATORY

The Medical Genomics Laboratory in the Department of Genetics at UAB, directed by Ludwine Messiaen, Ph.D, FACMG, is an academic non-for-profit clinical lab, CLIA and CAP certified. Our dedicated and experienced staff take pride in serving the patients and referring physicians the best we can.

We work closely with the UAB Neurofibromatosis clinic led by neurologists and geneticists Dr. Bruce Korf, Chairman of the Department of Genetics at UAB, and Dr. Lane Rutledge, Director of clinical services.

The clinic serves patients and their families dealing with the lifelong medical, psychological and social implications of the various forms of neurofibromatosis and RASopathies.

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